THE USE OF CULTURED CELLS WITH DEFECTS OF CITRULLINE METABOLISM IN DIAGNOSIS AND IN THE STUDY OF INTERCELLULAR COMMUNICATION

by

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ABSTRACT

Citrullinemia and argininosuccinic aciduria are two disorders resulting from defects in two consecutive enzymes of the urea cycle, argininosuccinate synthetase and argininosuccinate lyase. Fibroblast cell lines were derived from patients with these disorders and the diagnoses, which had been made on the basis of amino acid levels in plasma and urine, were confirmed by demonstrating that the cell lines were unable to incorporate ^{14}C-citrulline into protein. DNA from the argininosuccinate synthetase-deficient (ASS^-) cells was analysed by restriction enzyme digestion and hybridisation to a cDNA probe which had been cloned from human argininosuccinate synthetase mRNA. No defect in the patient's DNA could be demonstrated, indicating that no major deletions in the argininosuccinate synthetase genes were present in this patient.

Co-cultures of the ASS^- and argininosuccinate lyase-deficient (ASL^-) fibroblasts were able to incorporate ^{14}C-citrulline into protein at rates comparable to normal fibroblasts. This complementation did not require cell fusion, was dependent on cell contact, and was not the result of exchange of metabolites or enzymes via the culture medium. These results indicated that complementation occurred by the exchange of metabolites via intercellular junctions between the two cell types.

Co-cultures of ASS^- and ASL^- cells were used as an assay

system for measuring intercellular junctional communication. This allowed quantitation of the effects of pH and extracellular divalent cations on junctional communication.

Tumor promoters such as phorbol esters and organochlorine pesticides have been reported to inhibit intercellular junctional communication in other systems, and this inhibitory activity may be related to the mechanism of tumor promotion. The organochlorine pesticide 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT) was shown to be an inhibitor of junctional communication in ASS^-/ASL^- co-cultures. This inhibition was reversible, of rapid onset, and independent of extracellular calcium. The tumor-promoting phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA) also rapidly induced inhibition of junctional communication. However, co-cultures between Chinese hamster V79 cells, which are deficient in ASS, and ASL^- human fibroblasts were more sensitive to inhibition by TPA than the original ASS^-/ASL^- co-cultures. Refractoriness to TPA occurred following prolonged treatment with high concentrations of TPA.

Retinoic acid and other retinoids also inhibited junctional communication, and the inhibitory effects of retinoic acid and TPA were additive. The significance of these results in relation to the anti-tumor-promoting activity of retinoic acid is discussed.

It is concluded that co-cultures of ASS^- and ASL^- cells

constitute a useful system for providing quantitative measurements of intercellular junctional communication under a wide range of experimental conditions.

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1. Davidson, J.S., Baumgarten, I.M. and Harley, E.H. (1984). Metabolic cooperation between argininosuccinate synthetase and argininosuccinate lyase deficient human fibroblasts. Exp. Cell Res. 150, 367-378.

2. Davidson, J.S. and Harley, E.H. (1984). Complementation between argininosuccinate synthetase and argininosuccinate lyase deficient fibroblasts depends on intercellular communication. J. Inher. Metab. Dis. 7 (Suppl. 2), 141-142.

3. Davidson, J.S., Baumgarten, I.M. and Harley, E.H. (1984). Effects of extracellular calcium and magnesium on junctional intercellular communication in human fibroblasts. Exp. Cell Res., 155, 406-412

4. Davidson, J.S., Baumgarten, I.M. and Harley, E.H. Use of a new citrulline incorporation assay to investigate inhibition of intercellular communication by 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane. Cancer Res., In press.

5. Davidson, J.S., Baumgarten, I.M. and Harley, E.H. Effects of 12-O-tetradecanoylphorbol-13-acetate and retinoids on intercellular junctional communication measured with a citrulline incorporation assay. Carcinogenesis, In press.

CONTENTS

ABBREVIATIONS

ASA	:	Argininosuccinic acid
ASL	:	Argininosuccinate lyase
ASS	:	Argininosuccinate synthetase
ASL^-	:	Argininosuccinate lyase-deficient
ASS^-	:	Argininosuccinate synthetase-deficient
CPM	:	Counts per minute
DPM	:	Disintegrations per minute
TCA	:	Trichloroacetic acid
BME	:	Eagle's Basal Medium
MEM	:	Eagle's Minimal Essential Medium
EDTA	:	Ethylene diamine tetra-acetic acid
EGTA	:	Ethylene glycol tetra-acetic acid
EBV	:	Epstein-Barr Virus
FCS	:	Fetal Calf Serum
Ci	:	Curie ($2,2 \times 10^{12}$ disintegrations per minute)
Kb	:	Kilobases or kilobase pairs
tris	:	Tris (Hydroxymethyl) aminomethane
ATP	:	Adenosine triphosphate
DDT	:	1,1,1-Trichloro-2,2-bis(p-chlorophenyl)ethane
TPA	:	12-O-tetradecanoylphorbol-13-acetate
RA	:	Retinoic acid (all trans)
FA	:	Fluocinolone acetonide
DMSO	:	Dimethyl sulfoxide

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FIGURES

PART A

THE INVESTIGATION AND DIAGNOSIS OF UREA CYCLE DISORDERS USING CULTURED CELLS

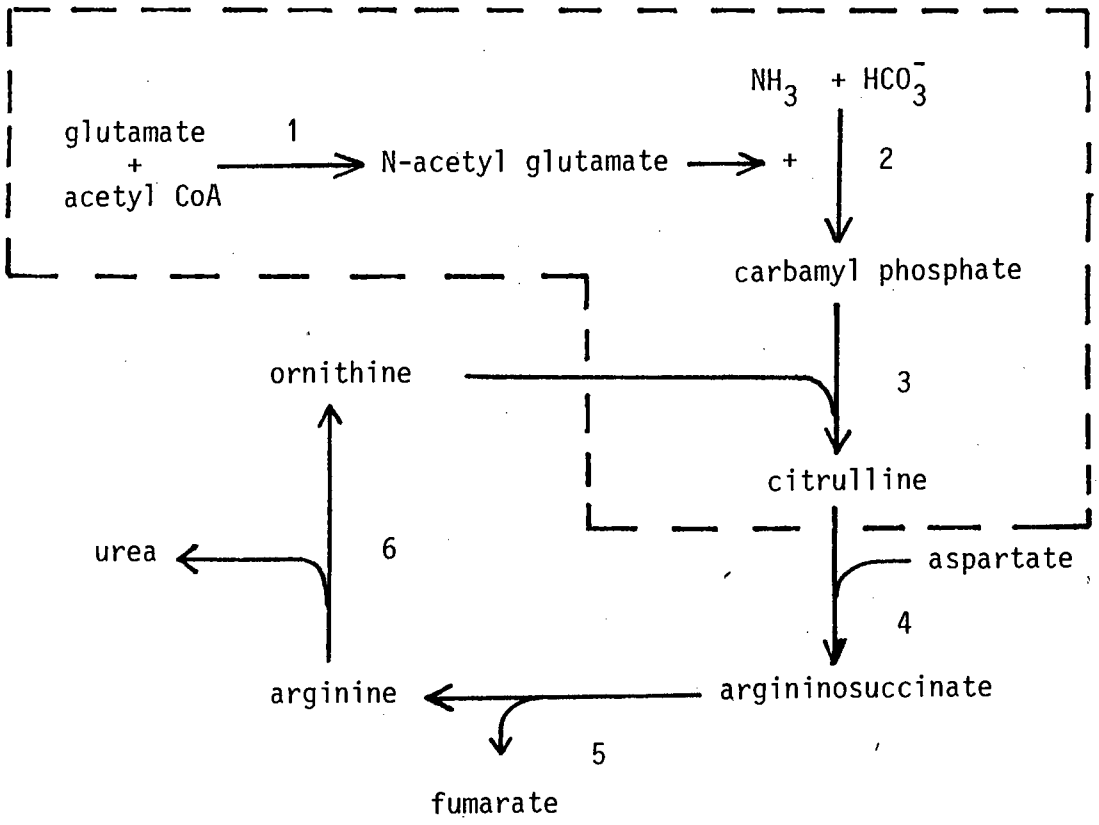
1. INTRODUCTION: DISORDERS OF CITRULLINE METABOLISM: CITRULLINEMIA AND ARGININOSUCCINIC ACIDURIA.

1.1. ENZYMES OF THE UREA CYCLE

The conversion of ammonia to urea in mammalian liver involves six enzymatic reactions, four of which form the urea cycle (Fig. 1). Deficiencies of each of these enzymes are well described (Walser, 1983). The work of this thesis focusses on two of these enzyme deficiencies: argininosuccinate synthetase (ASS; EC 6.3.4.5) deficiency which gives rise to the disorder citrullinemia (McKusick catalogue number 21570), and argininosuccinate lyase (ASL; EC 4.3.2.1) deficiency which results in argininosuccinic aciduria (McKusick catalogue number 20790).

Urea biosynthesis begins with the formation of carbamyl phosphate (CP) by the enzyme carbamyl phosphate synthetase (CPS). Two distinct carbamyl phosphate synthetases exist: a cytosolic form (CPS II) that can use glutamine or ammonia as substrate and produces CP as a precursor for pyrimidine synthesis, and a mitochondrial form (CPS I) that uses only ammonia and requires N-acetylglutamate for allosteric activation (Cheung and Raijman, 1981). The CP produced by CPS I is used almost entirely for urea production, except when mitochondrial CP levels become abnormally high. This occurs in deficiencies of the other urea cycle enzymes, and under these circumstances CP escapes into the cytosol and enters the pyrimidine synthetic pathway, resulting in

FIGURE 1



The urea cycle.

1. N-acetylglutamate synthetase
2. Carbamyl phosphate synthetase I
3. Ornithine transcarbamylase
4. Argininosuccinate synthetase
5. Argininosuccinate lyase
6. Arginase

The reactions enclosed by the dashed line are intramitochondrial; the remainder are cytoplasmic.

excretion of large amounts of orotic acid in the urine (Walser, 1983).

The activity of CPS I is regulated by the level of N-acetylglutamate (AGA) in the mitochondria. AGA is synthesised from acetyl CoA and glutamate in the mitochondria catalysed by N-acetylglutamate synthetase, an enzyme which itself is activated by high arginine levels (Coude et al., 1981; Kawamoto et al., 1982).

The formation of citrulline from carbamyl phosphate and ornithine is catalysed in the mitochondria by ornithine transcarbamylase. Citrulline leaves the mitochondrion and in the cytosol condenses with aspartate to form argininosuccinate (ASA) by the action of argininosuccinate synthetase (ASS). This reaction requires ATP and is inhibited by several amino acids including its own product argininosuccinate (Rochovansky et al., 1977; Tadaka et al., 1979).

Argininosuccinate is cleaved to form arginine and fumarate in the cytosol by argininosuccinate lyase (ASL). The fumarate is recycled by enzymes of the tricarboxylic acid cycle to form aspartate for the ASS reaction, and in this way a second nitrogen atom enters the urea synthetic pathway.

Arginine is cleaved in the cytosol to form urea and in the process regenerates the ornithine used by the CPS reaction.

Regulation of the urea cycle probably occurs at three levels: (a) the concentration of substrates (e.g. ammonia) will be regulatory where these concentrations are lower than the Km's of the respective enzymes, (b) allosteric effector molecules (e.g. AGA, arginine) are responsible for short-term regulation and (c) enzyme induction and repression allows long-term regulation (Schimke, 1964; Irr and Jacoby, 1978; Lin et al, 1982; Haggerty, 1982).

1.2. CITRULLINEMIA

At least 53 cases of ASS deficiency, or citrullinemia, have been reported (Walser, 1983). They may be divided into 3 groups. The neonatal type results from severe enzyme deficiency and most patients in this category have died in the neonatal period. The subacute type is less common, and results from a partial ASS deficiency. A late-onset type, confined to Japan, is also described.

All forms of citrullinemia present clinically with symptoms and signs of neurological dysfunction. In the severe neonatal form progressive neurological deterioration occurs, resulting in coma and death in most cases. In the subacute and late-onset forms of the disease, symptoms may be intermittent. Hyperammonemia is almost always present at some stage. The serum citrulline is elevated, and a markedly elevated serum citrulline in the absence of a raised level of argininosuccinate is pathognomonic of ASS deficiency. Glutamine and alanine are often elevated. This is probably secondary to the hyperammonemia, as it occurs in hyperammonemia from any cause (Walser, 1983). Urinary orotic acid is often elevated during hyperammonemic episodes. The excretion of large amounts of citrulline provides a pathway for ammonia detoxification, but at the expense of arginine. Consequently arginine supplementation is a rational therapy and is reported to be effective in some cases, when used in conjunction with protein restriction and sodium benzoate administration (Smith, 1981; Batshaw et al., 1982). Hippurate

is formed in the liver from benzoate and glycine, and is rapidly excreted by the kidneys, thus providing an additional pathway for removal of ammonia, which is utilised in the re-synthesis of glycine.

The clinical heterogeneity is paralleled by heterogeneity at the enzymatic level (Kennaway et al., 1975). However, no complementation has been observed between cells from different patients (Kennaway and Curtis, 1981), suggesting that all cases result from mutations in the same gene, probably the structural gene for ASS.

ASS deficiency is inherited as an autosomal recessive trait. The gene coding for ASS is on chromosome 9 (Carrit, 1977; Beaudet et al., 1982). A complementary DNA (cDNA) to the messenger RNA (mRNA) for ASS has been isolated and cloned (Su et al., 1981). The use of this cDNA as a molecular probe has permitted a detailed analysis of the molecular defect in some cases of citrullinemia (Su et al., 1982; Su et al., 1983). Thus the heterogeneity of citrullinemia at the clinical and enzymatic level has now been shown to be due, at least in part, to genetic heterogeneity at the DNA level.

1.3. ARGININOSUCCINIC ACIDURIA

Over 55 cases of ASL deficiency have been described (Walser, 1983). The neonatal type, due to severe ASL deficiency, is characterised by progressive neurological deterioration, resulting in coma and death in the neonatal period in most cases. Subacute and late-onset forms present predominantly with psychomotor retardation in the first or second year of life. Trichorrhexis nodosa, a hair abnormality, is present in many cases.

Argininosuccinate is markedly increased in the serum, and large quantities are excreted in the urine. Citrulline is elevated to a lesser extent, and hyperammonemia and orotic aciduria are present. As in citrullinemia, arginine deficiency may occur and patients may benefit from arginine supplementation (Shih, 1972), in addition to protein restriction, sodium benzoate administration, and dietary supplementation with alpha-keto analogues of essential amino acids. The alpha-keto acids are converted to their respective amino acids by a series of transamination and amination reactions, resulting in the removal of ammonia (Walser, 1983; Smith, 1981; Batshaw et al., 1982; Brusilow et al., 1984).

ASL deficiency is an autosomal recessive disorder, and the gene for ASL has been mapped to chromosome 7 (Naylor et al., 1978).

1.4. CITRULLINE METABOLISM IN CULTURED CELLS

Normal cultured human cells (fibroblasts, amniotic fluid cells, and lymphoblasts) do not contain ornithine transcarbamylase or carbamyl phosphate synthetase I and therefore do not have a functional urea cycle. They do possess ASS and ASL and can thus convert citrulline into arginine (Schimke, 1964; Tedesco and Mellman, 1967). These cultured cells can therefore be used in the laboratory diagnosis of citrullinemia and argininosuccinic aciduria, and two types of approach have been used.

The first approach is the measurement of ASS and ASL activity in cell extracts, which has shown that in patients with citrullinemia and argininosuccinic aciduria, the enzyme deficiency can be demonstrated in cultured cells. This has been used to confirm the diagnosis post-natally in these patients and to diagnose the disorders prenatally (Shih et al., 1969; Jacoby et al., 1972; Shih and Littlefield, 1970; Christiensen et al., 1980).

The second approach is by studying the metabolism of citrulline in intact, living cells. If normal cells are incubated with ^{14}C-citrulline, this is converted into ^{14}C-arginine which becomes incorporated into cell protein. Cultured cells from patients with citrullinemia or argininosuccinic aciduria are unable to convert citrulline to arginine at normal rates and hence show lower rates of

incorporation of ^{14}C-citrulline into protein (Tedesco and Mellman, 1967; Kennaway and Curtis, 1981). The advantages of this method over the direct enzyme assays are the simplicity of the procedure and the small number of cells required, a factor which is of special importance in prenatal diagnosis using amniotic fluid cells (Fleisher et al., 1979; Jacoby et al., 1981).

A less well-established technique for the prenatal diagnosis of these disorders is the measurement of citrulline and argininosuccinate in amniotic fluid (Goodman et al., 1973; Kamoun et al., 1983).

2. CASE REPORTS

2.1. CITRULLINEMIA: A CASE REPORT

A male child, G.A., was born to healthy non-consanguineous parents following a normal pregnancy and spontaneous vaginal delivery at term. Family history included the neonatal death of a sibling at 3 days ascribed to pneumonia, and there is a healthy male sibling now 4 years old. At birth the baby appeared normal and was discharged the following day. He returned to hospital within 3 days with a non-specific history of loose stools, feeding poorly and grunting respiration. He developed a twitch in his right arm, which progressed to a generalised seizure. He remained unconscious, maintaining an opisthotonic posture with extended arms and showing no response to painful stimuli. Other clinical features included diffuse rhonchi in both lung fields and peripheral oedema. Despite being ventilated he followed a rapidly downhill course with deterioration in peripheral circulation, and died within two days of admission without a diagnosis having been established. At autopsy the brain was very soft and oedematous and there was pulmonary congestion and haemorrhagic cystitis.

Biochemical Investigations

Initially serum lactate was 9.7 mM (ref. range 0.5 - 2.0) and pyruvate was 0.31 mM (ref. range 0.08 - 0.16). These levels were presumably post-ictal and were not maintained:

later measurements showed a lactate of 1.6 mM and pyruvate of 0.15 mM. Serum alanine determined enzymatically was elevated at 1.3 and 2.6 mM on two separate occasions (ref. range 0.2 - 0.4 mM). Plasma ammonia measured enzymatically was 440 µM, a ten-fold increase above normal. The serum urea was 2.9 mM which is well within the reference range of 2.1 - 6.7 mM, despite an elevated creatinine of 210 µM (ref. range 75 - 115 µM). Urine orotic acid measured by a colorimetric method (Kesner et al., 1975) was 1.2 mM. This represents a very great increase in orotic acid excretion, the normal value being less than 0.06 mM. The correct diagnosis only became apparent after death when the result of quantitative measurements on a Beckman amino acid analyser of the plasma and urine amino acids became available (Table 1). The plasma citrulline was 1.5 mM which is a 40 fold increase above the upper normal limit. Glutamine, alanine and lysine were elevated about three-fold above the upper normal limit, and arginine and cystine were significantly decreased. Four days post-mortem a sample of skin was removed from the forearm for fibroblast culture. The fibroblast cell line derived from this biopsy was designated F25.

TABLE 1

SERUM AMINO ACIDS IN THE PATIENTS WITH CITRULLINEMIA AND ARGININOSUCCINIC ACIDURIA

Serum amino acid levels (μM) in the patients. In the patient with argininosuccinic aciduria the isoleucine peak was obscured by the very large peak of argininosuccinate. ND = NONE DETECTED.

AMINO ACID	CITRULLINEMIA	ARGININOSUCCINIC ACIDURIA	NORMAL RANGE
Glutamine	1500	1300	370 - 500
Alanine	1200	750	200 - 470
Glycine	600	450	150 - 300
Proline	300	480	120 - 300
Valine	250	140	120 - 300
Lysine	950	250	80 - 250
Asparagine	200	140	40 - 180
Leucine	240	130	80 - 140
Threonine	160	120	60 - 170
Serine	260	170	100 - 160
Glutamic acid	300	400	40 - 140
Ornithine	90	50	50 - 140
Histidine	140	145	45 - 100
Arginine	25	25	40 - 100
½ Cystine	10	25	40 - 100
Tyrosine	150	125	30 - 80
Phenylalanine	80	65	40 - 80
Isoleucine	55	?	30 - 80
Aspartic acid	105	160	20 - 60
Hydroxyproline	40	6	14 - 50
Citrulline	1500	350	15 - 35
Methionine	75	100	15 - 35
α-amino butyrate	70	80	10 - 26
Argininosuccinate	ND	2000	ND

2.2. ARGININOSUCCINIC ACIDURIA: A CASE REPORT

The patient, B.J., was born to normal parents. Their previous child had died in the neonatal period of an undiagnosed disorder. There was no other family history of note. Rapid neurological deterioration associated with hyper-ammonemia occurred during the first few days after birth. Urinary orotic acid was 0.7 mM. Serum amino acids analysed by cation-exchange column chromatography are shown in Table 1. Both serum and urine from the patient showed 3 very large peaks which were absent from normal serum and urine. Authentic barium argininosuccinate (Sigma) which had been allowed to stand in aqueous solution for 3 hours at room temperature, chromatographed as 3 peaks in identical positions to the peaks in the patient's serum and urine. Two of the peaks were present only in very small amounts in freshly-prepared argininosuccinate solutions: these species are the anhydrides of argininosuccinate which form spontaneously in aqueous solutions (Westall, 1980). The concentration of argininosuccinate was 2.0 mM in the serum and 14.0 mM in the urine. The baby died aged 8 days. A skin biopsy was kindly provided by Dr K. Sprenger and Prof. M. Berger, Dept. of Chemical Pathology, Red Cross War Memorial Children's Hospital, Cape Town, and the fibroblast cell line derived from it was designated F199. Lymphocytes from 5 ml of this patient's blood were transformed with Epstein-Barr virus, and the resulting lymphoblast cell line was designated L199 (see Section 3.1.2).

3. MATERIALS AND METHODS

3.1. CELL CULTURE METHODS

3.1.1. Establishment of fibroblast cultures

Fibroblast cultures were either retrieved from storage in liquid nitrogen, or grown from fresh skin biopsies. Biopsies were taken as follows: a piece of skin, usually from the ventral surface of the forearm, measuring about 2mm x 2mm was removed by inserting a sterile hypodermic needle into the dermis, lifting the skin away from the arm, and undercutting the needle with a sterile blade. The biopsy was immediately placed in Ham's F10 medium. Under sterile conditions the fragment of skin was cut up into 10 to 20 smaller fragments, which were placed under glass coverslips in plastic petri dishes (35 mm diameter, Falcon). After several days, when cells were seen to be growing under the coverslips, the coverslips were inverted and placed in different petri dishes. Both sets of dishes were kept until confluent and then trypsinised into 25 cm^2 Falcon plastic flasks.

The origins of the fibroblast cell lines used are given below:

F 25 - patient with citrullinemia (G.A.)

F 199 - patient with argininosuccinic aciduria (B.J.)

FM 199 - mother of B.J.

FF 199 - father of B.J.

F 139 - Normal control

F 140 - " "

FG - " "

F 200 - " "

Amniotic fluid cell cultures were kindly provided by Mrs E.M. Petersen, Dept. of Human Genetics, University of Cape Town. None of the control cultures were known to have inherited metabolic disorders. The indication for amniocentesis was maternal age in most cases, and the karyotype was normal in all.

3.1.2. Establishment of lymphoblast cultures

Five to 10 ml of heparinised whole blood was carefully layered on to 7 ml of Ficoll-Paque in a sterile plastic tube. After centrifuging in a swing-out rotor for 20 minutes at 1000 g, the white cell layer at the interface between the Ficoll and the plasma was removed with a pasteur pipette and mixed with 5-10 ml Ham's F10 medium. After re-centrifuging, the supernatant was decanted and the cell pellet resuspended in 1.5 - 4 ml transforming medium (see below), depending on the white cell yield. Transformation was usually apparent by 7 to 21 days. Cells were examined daily and small volumes (0.5 ml) of fresh medium were added every 3 or 4 days, until transformed cells became visible, after which fresh medium was added in larger amounts and more frequently.

Transforming medium was prepared as follows: Two 75 cm^2 flasks of Marmoset cells producing Epstein-Barr virus (the B95/8 cell line) were grown to confluence and allowed to condition their medium for 4 days. The medium was decanted, centrifuged to remove debris, filtered (pore size 0.2 µm), diluted with an equal volume of Ham's F10 medium containing

15% foetal calf serum (FCS) and aliquotted into 20 ml amounts.

3.1.3. Maintenance of cell cultures

Cells were grown in plastic tissue culture flasks (Falcon) in 5% CO_2 at 37 degrees centigrade. Cultures were routinely maintained on Ham's F10 medium (Flow Laboratories) containing 15% FCS without antibiotics, which was changed twice weekly. One to three days before using the cells for labelling studies, the medium was changed to Eagles Basal Medium (BME, Flow Laboratories) containing 10% FCS. This was done to decrease the intracellular pool of arginine so that ^{14}C-citrulline would be incorporated with high efficiency.

Subculturing of fibroblasts was done by decanting the growth medium, washing once with trypsin/EDTA (Adams, 1980), and incubating the cells at 37^{o}C with intermittent shaking until they were detached from the substratum. The resulting cell suspension was diluted with an appropriate volume of growth medium and divided among the required number of flasks. The split ratio was usually 1 into 3.

All cell lines were regularly checked for mycoplasma contamination by fluorescent staining with Hoechst 33258.

3.2. INCORPORATION OF RADIO-ISOTOPES INTO TCA-INSOLUBLE MATERIAL

3.2.1. Labelling conditions

The following isotopes were obtained from the Radiochemical Centre, Amersham, U.K.:

Isotope	Specific Activity
L-|carbamoyl-^{14}C| citrulline	55 mCi/mmol
L-|4,5-^{3}H| leucine	120-190 Ci/mmol
|U-^{14}C| leucine	56 mCi/mmol
L-phenyl |2,3-^{3}H| alanine	29.1 Ci/mmol
L-|5(n)-^{3}H| arginine hydrochloride	19 Ci/mmol

Isotopes were added to the entire quantity of labelling medium to be used for each experiment and not individually to each dish or flask of cells. This eliminated errors due to inaccurate pipetting of isotopes between dishes. Unlabelled citrulline was not added except where stated. The following media were used for labelling:

Medium BF: Eagles Basal Medium (BME) with 10% Foetal calf serum

Medium B : BME without serum

Medium A : Eagles Minimal Essential Medium without arginine (No serum was added)

Medium Y :

NaCl	130 mM
KCl	5 mM
Hepes (pH 7.4)	15 mM
D-glucose	8.3 mM
phenol red	4 mg/l

Medium Z : As for medium Y, with the addition of $CaCl_2$ (1 mM) and $MgCl_2$ (1 mM)

All incubations were at 37°C, using 1.5 ml or 2 ml of labelling medium in 60 mm plastic dishes or 25 cm^2 plastic flasks (Falcon or Greiner). When media BF, B or A were used, cells were incubated in 4% CO_2. When media Y or Z were used, incubations were without CO_2.

Incubation periods ranging from 1 to 22 hours were used. The duration of the incubation period is given for each experiment in the Results section. The duration of the labelling period was determined largely by the arginine concentration in the labelling medium. BME contains 0.1 mM arginine and in order to obtain sufficient incorporation of ^{14}C-citrulline into cell protein, a minimum labelling period of 4 hours was required, using ^{14}C-citrulline at 0.5 µCi/ml. Shorter labelling periods in this medium could only be achieved by using ^{14}C-citrulline at higher concentrations, which was only undertaken when strictly necessary on account of the expense of the isotope. In later experiments media A, Y or Z were used which do not contain arginine. This allowed lower concentrations of ^{14}C-citrulline to be used. Immediately before labelling in media A,Y or Z, the cells were washed once with PBS, to remove unlabelled arginine from the residual growth medium. Where cells had been grown in Ham's F10 medium, the medium was changed to BME at least 20 hours before labelling, in order to decrease

the intracellular pool of unlabelled arginine (Ham's F10 contains 1.0 mM arginine, whereas BME contains only 0.1 mM).

Isotopes were used at concentrations ranging from 0.125 μCi/ml to 2 μCi/ml. The actual concentrations used are given with the results of each experiment.

Unlabelled citrulline was not added to the labelling medium, except where indicated in the results section.

3.2.2. Preparation of TCA-insoluble material

After the labelling period the cells were washed 3 times with PBS and 3 times with 10% TCA. In the earlier experiments, fibroblasts were trypsinised and washed by repeated centrifugation and resuspension. This procedure was also used for suspension cultures of lymphoblasts, and for those experiments using fibroblasts where adhesion to the substratum was prevented. In later experiments a simpler procedure of washing the cells while still attached to the dishes was adopted. After the final TCA wash, the dishes were drained and the cell residue was dissolved in 1.3 ml of 0.1 N NaOH. Of this solution, 0.1 ml was used for protein determination (Section 3.2.3) and the remainder was neutralised with 66 μl of 10% glacial acetic acid. This was necessary to prevent spurious counts due to chemiluminescence. One ml of the neutralised solution was mixed with 10 ml Instagel scintillant (Packard Instrument Co., Illinois, USA) and counted in a Beckman LS-250 scintillation spectrometer. The window

settings were such that ^{3}H counts were excluded from the "^{14}C" window, and less than 7% of ^{14}C counts appeared in the "^{3}H" window. To correct for the ^{14}C counts appearing in the "^{3}H" window, a quench curve was determined using commercial standards (Beckman). Disintegrations per minute (DPM) were calculated from this quench curve using the counting efficiency determined by external standardisation. Background counts have been subtracted from all data shown.

The adequacy of the washing procedures described above in removing radioactivity not incorporated into TCA-insoluble material was monitored frequently by including the cell line F25 in the experiment. This cell line incorporates only negligible amounts of ^{14}C-citrulline, but shows normal incorporation of ^{3}H-leucine or ^{3}H-phenylalanine (see Table 2). Consequently a failure to remove unincorporated ^{14}C-citrulline would result in an increase of ^{14}C counts in these cells and would be easily detected.

Results were expressed as the ratio of ^{14}C-DPM to ^{3}H-DPM (usually having labelled the cells with ^{14}C-citrulline and either ^{3}H-leucine or ^{3}H-phenylalanine).

3.2.3. Determination of cell proteins

Cell protein was determined using the Bradford method (Bradford 1976). After washing the cells and precipitating with TCA (Section 3.2.2) the TCA insoluble material was dissolved in 1.3 ml of 0.1 N NaOH. A 0.1 ml aliquot of this

solution was added to 2.1 ml water and 0.4 ml Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Richmond, California), in a standard 10 mm path cuvette. The contents of the cuvette were immediately mixed and the absorbance at 595 nm was read after 30 to 60 seconds in a Varian Techtron model 635 spectrophotometer against a blank containing water instead of cell extract. Bovine serum albumin at concentrations of between 100 and 400 µg/ml was used as standard.

3.3. ANALYSIS OF GENOMIC DNA FROM CITRULLINEMIC CELLS

3.3.1. DNA isolation from cultured cells

Approximately 2.5 x 10^7 fibroblasts or lymphoblasts were harvested and washed twice with normal saline by centrifugation at 2000 g for 10 minutes at room temperature. The pelleted cells were homogenised on ice in 30-60 ml cold lysis buffer (0.32 M sucrose, 5 mM $MgCl_2$, 1% triton X-100 and 10 mM tris HCl, pH 7,6), using a Dounce homogeniser.

The nuclei were pelleted by centrifugation at 2500 g for 20 minutes. The pellets were resuspended in a total volume of 8 ml of 75 mM NaCl, 25 mM EDTA (pH 8,0) with subsequent addition of 0.8 ml 10% SDS. The solutions were mixed, 1 mg of pronase (Sigma) was added, and incubated at 37°C for 3 hours. The pronase digestion was stopped by placing the samples on ice, and 0.5 ml 5 M sodium perchlorate solution was added, followed by extraction with 8 ml phenol : chloroform (1:1, saturated with 1 mM EDTA, 10 mM tris HCl, pH 7.6). The aqueous and organic phases were separated by centrifugation at 2500 g for 10 minutes at 10°C. The upper aqueous phase was re-extracted with an equal volume of chloroform : octanol (24:1). The samples were centrifuged at 2500 g for 10 minutes at 10°C to separate the two phases. The aqueous phase was pipetted off and the nucleic acid was precipitated by the addition of two volumes of ice-cold absolute ethanol to the aqueous phase. After

centrifugation, the nucleic acid pellet was dissolved overnight in 1 ml of 1 mM EDTA, 10 mM Tris HCl pH 7,6.

To the nucleic acid solution 0.10 ml (1/10th volume) 4 M NaCl was added, followed by 0.05 mg heat-treated RNAse (Sigma). The solution was incubated at 37°C for 1 hour. After digestion, 2 ml sterile distilled water was added and the solution extracted with an equal volume of chloroform : octanol (24:1) until the interface was clear. The samples were centrifuged at 2500 g for 10 minutes at 10°C to separate the aqueous and organic phases and the DNA was precipitated from the aqueous phase by the addition of two volumes of ice-cold absolute ethanol. The precipitate was washed twice in 70% ethanol to remove any remaining salt. The DNA pellet was dried under a vacuum and redissolved, by gentle mixing, in 1 ml sterile distilled water at 4°C overnight.

The final DNA concentration was estimated by the determination of the absorbance at 260 nm, assuming that a solution containing 1 mg/ml DNA has an absorbance of 20 O.D. units in a 1 cm path cuvette. The DNA samples were stored at -20°C until further use.

3.3.2. Preparation of pAS-1 plasmid

The pAS-1 plasmid in E. coli strain RR1 was kindly supplied by Dr T.S. Su, Dept. of Cell Biology, Baylor College of Medicine, Houston, Texas. The pAS 1 plasmid contains a

DNA sequence complementary to the mRNA for human argininosuccinate synthetase (Su et al., 1981).

On the first day, the bacteria were innoculated into 20 ml of L-broth (10 g Difco tryptone, 5 g Difco Bacto yeast extract, 5 g NaCl and 1 g glucose in 1 litre water and autoclaved at 15 psi for 15 minutes) and 20 µl of a 20 mg/ml solution of tetracycline (Sigma) was added. The culture was grown overnight while shaking at 37°C.

On the second day, 5 ml of the starter culture was innoculated into four 500 ml Erlenmeyer flasks containing 250 ml L-broth and 0.25 ml tetracycline (20 mg/ml). The flasks were incubated at 37°C with constant rotation until the absorbance at 660 nm of the medium was between 0.60 and 1.00 units (i.e. the cells present were in the logarithmic growth phase).

Chloramphenicol (Sigma), (final concentration = 0.18 mg/ml) was then added and the subsequent amplification of the plasmid allowed to proceed overnight with gentle shaking.

The bacterial cells were harvested by centrifugation at 1500 g for 10 minutes at 4°C and the resultant pellets resuspended in 7 ml 25% sucrose, 50 mM Tris HCl, pH 8.0. Freshly prepared egg-white lysozyme (1.6 ml of a 25 mg/ml solution) was added and the solution gently mixed on ice for 5 minutes. To this, 1.3 ml lytic mix (2% Triton X-100, 6 mM EDTA and

50 mM Tris HCl, pH 8.0) was added and the mixing continued for a further 20 minutes, on ice. During this period the cells lysed to form a viscous mass. The bacterial cell debris carrying with it the bulk of the bacterial DNA was pelleted by centrifugation at 18000 g for 1 hour at 4°C.

The clear, straw-coloured supernatant was decanted and extracted twice with an equal volume of phenol : chloroform (1:1) followed by two extractions with chloroform : octanol (24:1). To the final clear aqueous phase 1/10 volume of 4 M NaCl was added, followed by two volumes of ice-cold absolute ethanol and the nucleic acid precipitated overnight at -20°C. The precipitate was recovered by centrifugation at 2000 g for 10 minutes at 4°C, washed in 70% ethanol and dried under a vacuum. The resultant pellet was redissolved in 3 ml 1 mM EDTA, 10 mM Tris HCl, pH 7.6 at 4°C for at least 1 hour. T1-RNAse (Sigma) was added to a final concentration of 20 µg/ml and the solution incubated at 37°C for 30 minutes. The solution was extracted with an equal volume of chloroform : octanol (24:1) and the DNA in the aqueous phase precipitated by addition of 1/10 volume 4 M NaCl and two volumes of ice-cold absolute ethanol. The precipitate was recovered by centrifugation at 2000 g for 10 minutes at 10°C, washed twice in a small volume of 70% ethanol and dried under a vacuum. The DNA was then redissolved in 0.25 - 0.50 ml TE buffer (1 mM EDTA, 10 mM Tris HCl, pH 8.0).

The plasmid DNA was further purified by chromatography on a

Sephadex G-200 column. Twelve fractions of 0.50 ml each were collected into sterile Eppendorf tubes. Aliquots of these fractions were electrophoresed on a 0.80% agarose gel to identify the fractions containing the recombinant plasmid. They were pooled and scanned from A_{220} to A_{310} to determine the DNA concentration. The intact plasmid was used for nick-translation (Section 3.3.6).

3.3.3. Restriction endonuclease digestion of total human DNA

Ten micrograms of DNA were digested with 30 units of restriction endonuclease (BRL, Boehringer-Mannheim and New England Nuclear Laboratories) in the presence of the enzyme buffers (composition as recommended by the manufacturers) and nuclease-free BSA (Sigma) at a final concentration of 100 µg/ml. The volume in which the DNA was digested was made up to 0.15 ml with sterile distilled water. The digestion was allowed to proceed for a minimum of 6 hours at 37°C and was terminated by the addition of 1/10th volume of 100 mM EDTA, pH 8.0. To this, 1/10th volume of 4 M NaCl was added and the DNA precipitated by the addition of two volumes of ice-cold absolute ethanol. The precipitated DNA was recovered by centrifugation, washed in 70% ethanol and dried under vacuum. The DNA samples were dissolved in 34 µl sterile distilled water with gentle mixing at 4°C for at least 12 hours prior to electrophoresis.

3.3.4. Agarose gel electrophoresis

To the dissolved DNA, 6 µl orange-G/Ficoll (1% w/v orange-G, 20% Ficoll, 20 mM EDTA) and 4 µl electrophoresis buffer (40 mM acetate, 2 mM EDTA and 40 mM Tris HCl, pH 7.6) was added. A 0.8% agarose gel was prepared by boiling 2 g agarose in 250 ml of the above buffer. Ethidium bromide to a final concentration of 1 µg/ml was added prior to pouring the gel onto a sealed 20 cm by 20 cm glass plate in the gel apparatus. The gel was allowed to set for at least one hour. Prior to loading, the DNA samples were incubated at 37°C for 15 minutes to ensure that the DNA was completely dissolved. The gel apparatus was filled with electrophoresis buffer and the wells flooded. The DNA samples were carefully pipetted into the wells below the surface of the buffer and were run into the gel at 25 volts, 20 milliamps for 10 minutes. The voltage was adjusted to 40 volts and electrophoresis performed for at least 18 hours or until the orange-G front had completely disappeared off the end of the gel.

For optimal resolution of similarly sized bands the gel apparatus was placed at 4°C, the buffer circulated and the DNA electrophoresed for 24 to 30 hours.

Upon completion of the electrophoresis, the gel was viewed on an ultra-violet light box and photographed with a ruler in line with the molecular weight markers. The reciprocal of the mobility of the molecular weight markers was plotted

against their known fragment sizes (kilobases). The sizes of the DNA fragments were read from the graph after autoradiography.

The DNA was denatured by gentle agitation of the gel in 300 ml 0.5 M NaOH, 1.5 M NaCl for 3 hours. The gel was rinsed twice in distilled water and neutralized in 0.5 M Tris, 20x SSC (3 M NaCl, 0.3 M Tri-sodium citrate, pH 7.4) for a further 2 hours. The gel was then ready for the Southern blotting procedure.

3.3.5. Southern blotting procedure

This was performed essentially according to the method of Southern (1975).

A platform consisting of four Bijou bottles, placed on their sides, and a 20 cm x 20 cm glass plate was set up in a large developing tray (8 cm deep). A piece of Whatman no. 1 filter paper, 24 cm x 24 cm, was cut and folded over the glass plate to act as a wick. 20x SSC was poured onto the filter paper to within 2 cm of the plate. Air bubbles between the filter paper and the glass plate were rolled out with a small glass rod. The pre-treated gel was slid onto a filter paper on the glass plate, ensuring that no air bubbles were trapped between the gel and the support.

A 20 cm x 20 cm nitrocellulose sheet (Schleicher and Schuell BA 85) was wet by flotation in 100 ml 2x SSC and lowered

onto the gel, taking care to remove air bubbles. Strips of cling film were placed from the outer edges of the gel to the edges of the tray to ensure diffusion of the 20x SSC through the gel and to prevent evaporation of the 20x SSC during the transfer. Two sheets of Whatman no. 1 filter paper (20 cm x 20 cm) were wet by flotation in the 2x SCC and placed on top of the nitrocellulose avoiding air bubbles. A box of tissues was divided in two and placed side by side on top of the filter papers. A 20 cm x 20 cm glass plate, with 4 x 125 g weights, was placed on top of the tissues and the whole assembly was placed at 4°C for 30 hours. The level of the 20x SSC was monitored and the saturated tissues replaced with dry ones when necessary.

After DNA transfer had been effected, the wells of the gel were marked on the overlying nitrocellulose, which was then cut into strips compatible with the size of the hybridization box. The filters were lifted from the gel and rinsed for 10 minutes in 2x SSC. They were blotted dry and the DNA was baked onto the nitrocellulose at 80°C for 2 hours. The filters could be stored indefinitely at 4°C at this stage.

3.3.6. Nick-translation of the pAS1 plasmid

In order to prepare a highly radioactive probe, 0.5 µg of plasmid DNA was incubated for 90 minutes at 16°C in a solution containing 20 µM dTTP, dATP, dGTP, 60 µCi ^{32}P-dCTP (specific activity = 3000 Ci/mmol), 5 mM $MgCl_2$, 10 mM 2-mercapto-ethanol, 10 µg/ml nuclease-free BSA (Sigma) and

50 mM Tris HCl, pH 7.6 (Rigby et al., 1977). Two hundred picograms of DNAse 1 and 2 units of DNA polymerase (BRL Nick-Translation Kit) were added and the final reaction volume made up to 50 µl with sterile distilled water. The reaction was terminated by the addition of 100 µl of a stop-mix solution (10 mM EDTA, 0.5% SDS, 10 mM Tris HCl, pH 7.6) and placed on ice. The solution was extracted with an equal volume of phenol : chloroform (1:1), the phenol phase re-extracted with TE buffer and the pooled aqueous phases loaded onto a 5 cm x 0.5 cm column of Sephadex G-50 (medium). Twelve fractions (150 µl each) were collected and 1.5 µl aliquots counted for Cerenkov radiation with a counting efficiency of 40%. The fractions of the excluded peak were pooled and the specific activity (in dpm/µg) of the labelled DNA calculated as follows:

$$\text{Specific activity} = 2\,(\text{sum of the dpm in the pooled fractions}) \times 100/0.4 = \text{dpm}/\mu\text{g}$$

The radio-labelled DNA was denatured by incubation in a boiling water-bath for 5 minutes, followed by rapid cooling on ice to prevent re-annealing of the DNA strands.

3.3.7. Hybridization of the probe to genomic DNA

The nitrocellulose filters were wet by flotation in 3x SSC and washed in the following solutions:

1. 3x SSC for 30 minutes at 65°C,
2. 3x SSC, 5x Denhardts* solution for 1 hour at 65°C,

3. 3x SSC, 5x Denhardts solution, 0.1% SDS, 10 µg/ml polyadenylic acid and 50 µg/ml single-stranded Herring sperm DNA for 1 hour at 65°C in a shaking water-bath.

*Denhardts solution (10x):

2% w/v bovine serum albumin (Sigma fraction V)
2% w/v Ficoll (type 400) (Pharmacia)
2% w/v polyvinylpyrrolidone (Sigma PVP-360)

Each solution was pre-heated to 65°C and the strips of nitrocellulose filters transferred individually with a pair of Millipore forceps.

The filters were hybridized in a hybridization box (designed by A. Jeffries, Leicester University). Ten millilitres of the wash solution no. 3 were pipetted into the hybridization box, the ^{32}P-labelled single-stranded DNA probe added and the filter strips individually transferred to the hybridization solution, ensuring that no air bubbles were trapped between the filters. The hybridization box was tightly sealed and incubated in a shaking water-bath at 65°C for at least 24 hours.

Upon completion of the hybridization, the filters were washed at 65°C to remove unbound probe as follows:

1. 3x SSC, 5x Denhardt's solution, 0.1% SDS for 4 x 2 minutes and then 2 x 30 minutes
2. 1x SCC, 0.1% SDS for 2 x 30 minutes
3. 0.5x SSC, 0.1% SDS for 30 minutes

The amount of radioactivity remaining on the filters was monitored with a Geiger counter, and if significantly above background, the stringent (low ionic strength) wash was repeated. The filters were blotted dry with Whatman no. 1 filter paper and further dried at 37°C for 30 minutes. They were re-assembled onto cardboard, covered with cling-film and autoradiographed using Kodak X-omat MA X-ray film and a Dupont intensifying screen. The X-ray film was exposed at -70°C for 1-10 days as required. The autoradiograph was developed in Kodak X-ray developer for 3 minutes, rinsed in 2% glacial acetic acid for 20 seconds and fixed in Kodak fixative for 6 minutes. The autoradiograph was then washed in running water for 15 minutes and dried.

The distances of the bands from the origin of migration were measured and the sizes of the DNA fragments in the respective bands were calculated from the standard curve.

3.4. MISCELLANEOUS METHODS

Serum and urinary amino acids were measured by cation exchange chromatography on a Beckman Model 121-M amino acid analyser.

Urinary orotic acid was measured by the method of Kesner et al. (1975).

The levels of serum ammonia, lactate, pyruvate, alanine (enzymatic method), urea, and creatinine were measured by the Chemical Pathology Routine Laboratory at Groote Schuur Hospital, Cape Town, by standard methods.

4. RESULTS

4.1. CITRULLINE INCORPORATION BY CULTURED FIBROBLASTS AND AMNIOTIC FLUID CELLS

The results of a typical experiment in which incorporation of ^{14}C-citrulline and ^{3}H-leucine into TCA-insoluble material by fibroblasts was measured is shown in Table 2.

Cumulated results from several experiments of this type are shown in Figure 2. The ^{14}C DPM/^{3}H DPM ratios are plotted on a logarithmic scale. The mean ratio in control cells (Fig. 2A) was 170-fold higher than the mean ratio in the F25 cells (Fig. 2B) and 100-fold higher than the mean ratio in the F199 cells (Fig. 2E). Thus both of these cell lines have a virtually complete block in the conversion of citrulline to arginine.

The cells of the parents of the patient with ASL-deficiency (Fig. 2F) showed citrulline incorporation within the range of normal fibroblasts.

The ^{14}C-DPM/^{3}H-DPM ratios obtained in normal amniotic fluid cell cultures are shown in Fig. 2C. The normal range in amniotic fluid cells was very wide, the cultures with the lowest ratios having only 3% of the citrulline-incorporating activity of the cultures with the highest ratios. Despite this wide range, even the cultures showing the lowest citrulline incorporation rates were clearly separated from the ASS$^-$ and ASL$^-$ fibroblasts.

TABLE 2

CITRULLINE INCORPORATION RELATIVE TO LEUCINE IN THREE NORMAL FIBROBLAST LINES AND IN ASS-DEFICIENT AND ASL-DEFICIENT FIBROBLASTS

CELL LINE	^{3}H DPM	^{14}C DPM	$\frac{^{14}C \text{ DPM}}{^{3}H \text{ DPM}} \times 100$
F139	32213	3257	10.1
(control)	28555	2798	9.8
F200	15483	1667	10.8
(control)	16321	1792	11.0
FG	50836	5192	10.2
(control)	57721	5790	10.0
F25	39128	10	0.03
(ASS^{-})	33167	21	0.06
F199	24236	21	0.09
(ASL^{-})	25211	18	0.07

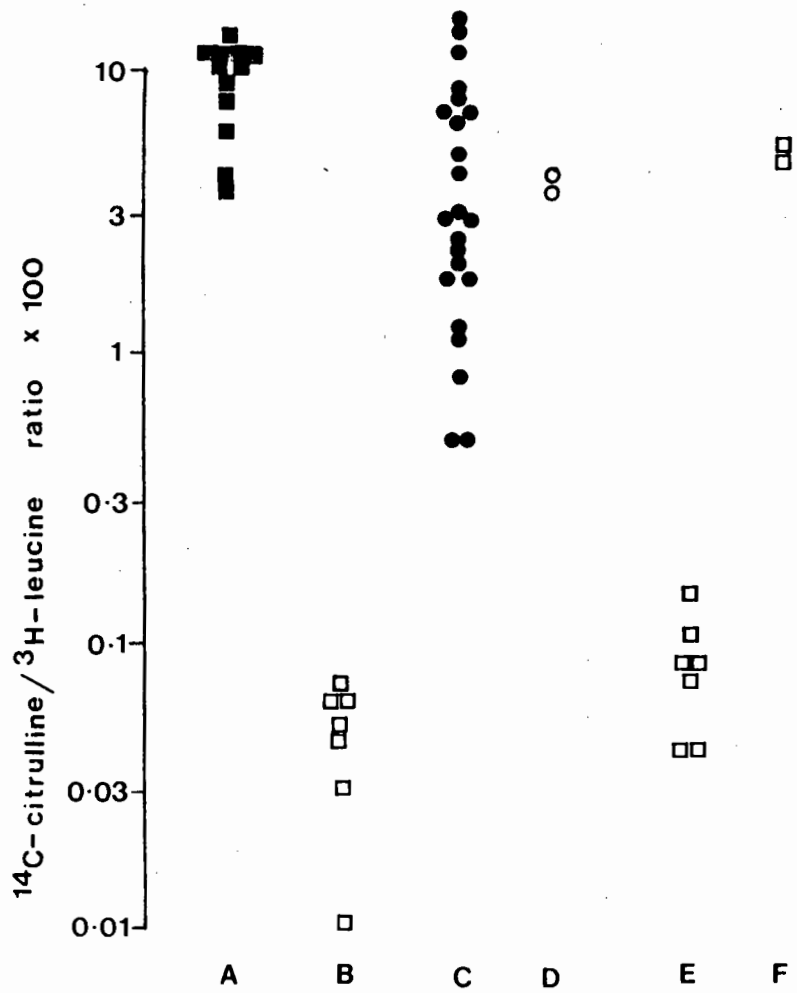
Labelling medium : B

Labelling period : 5 hours

^{14}C-citrulline : 0.5 µCi/ml

^{3}H-leucine : 0.5 µCi/ml

FIGURE 2



^{14}C-citrulline incorporation relative to ^{3}H-leucine in fibroblasts and amniotic fluid cells.

A. Normal fibroblasts
B. Fibroblasts from the patient with citrullinemia
C. Normal amniotic fluid cells
D. Amniotic fluid cells from the foetus (sibling of B) at risk for citrullinemia
E. Fibroblasts from the patient with argininosuccinic aciduria
F. Fibroblasts from the mother and father of E.

Labelling conditions were as described in Table 2.

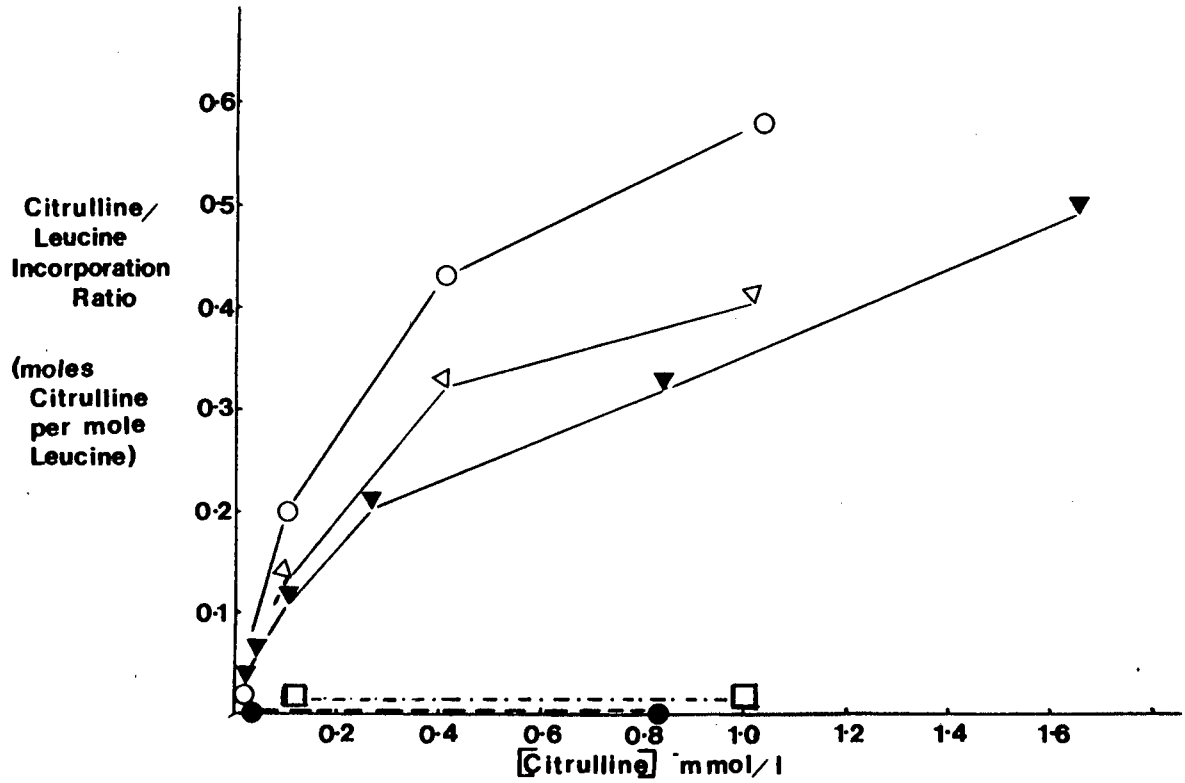
The mother of the citrullinemic child from which cell line F25 was derived subsequently became pregnant, and the amniotic fluid cells from this pregnancy showed citrulline incorporation within the normal range (Fig. 2D). On the basis of this result the pregnancy was allowed to continue to term and a healthy female infant was delivered.

4.2. CITRULLINE INCORPORATION IN RELATION TO EXTRACELLULAR CITRULLINE CONCENTRATION

The citrulline incorporation assays described above were performed without adding unlabelled citrulline to the labelling medium, so that the total citrulline concentration was contributed entirely by the ^{14}C-citrulline, resulting in a final citrulline concentration of 0.01 mM. The relationship of citrulline incorporation to extracellular citrulline concentration was examined in 3 normal fibroblast lines and in the ASS^- and ASL^- cells (Fig. 3).

The normal cells showed partially saturable kinetics with half-maximal citrulline incorporation at a citrulline concentration of about 0.4 mM. Neither the ASS^- nor the ASL^- cells showed any increase in citrulline incorporation at higher citrulline concentrations.

FIGURE 3



Citrulline incorporation relative to leucine in relation to extracellular citrulline concentration.

Labelling medium : BF
Labelling period : 20 hours
^{14}C-citrulline : 0.25 µCi/ml
^{3}H-leucine : 0.125 µCi/ml

Varying amounts of unlabelled L-citrulline were added to the culture flasks to give the final concentrations shown. The molar incorporation ratio (moles citrulline incorporated per mole of leucine incorporated) was calculated as follows:

$$\text{CIT/PHE Molar Ratio} = \frac{^{14}\text{C-DPM}}{^{3}\text{H-DPM}} \times \frac{\text{SA (leu)}}{\text{SA (cit)}}$$

where SA (leu) and SA (cit) are the final specific activities of the isotopes in the labelling medium.

The symbols represent the following fibroblast cell lines:

○	F 83	Normal control fibroblasts
▽	F 27	Normal control fibroblasts
▼	F 108	Normal control fibroblasts
●	F 25	ASS$^-$ (citrullinemia)
□	F 199	ASL$^-$ (argininosuccinic aciduria)

4.3. PRECISION OF THE DOUBLE-LABELLING METHOD

In all experiments in which cells were labelled with ^{14}C-citrulline and ^{3}H-leucine or ^{3}H-phenylalanine, the ^{14}C DPM/^{3}H DPM ratio was found to be measurable with high precision within experiments (for example see Table 2). The mean difference between duplicates in 26 pairs of duplicate flasks in 6 different experiments was 5.3% $\pm$ 4.4 (mean $\pm$ S.D.).

4.4. MEASUREMENT OF VARIATION IN ARGININE INCORPORATION

It could be argued that if variations occurred in the rate of arginine incorporation between cell lines or with different culture conditions, the rate of citrulline incorporation would be altered. Therefore differences in citrulline incorporation might not necessarily reflect differences in citrulline metabolism, but merely differences in arginine metabolism.

For this reason arginine incorporation relative to leucine was measured in the ASS^- and ASL^- cell lines and in two normal cell lines (Table 3). The incorporation ratio of ^{14}C-leucine relative to ^{3}H-arginine was similar in all the cell lines tested, and varied by less than 8% with different degrees of confluence of the cultures. Differences in the incorporation ratio of ^{14}C-citrulline to ^{3}H-leucine should therefore reflect differences in the rate of conversion of citrulline to arginine.

TABLE 3

^{14}C-LEUCINE INCORPORATION RELATIVE TO ^{3}H-ARGININE IN NORMAL, ASS$^-$ AND ASL$^-$ FIBROBLASTS

Cells were labelled at two cell densities, confluent (C) and sub-confluent (S).

CELL LINE	CONFLUENCE	$\frac{^{14}C\ DPM}{^{3}H\ DPM}$
F139 (control)	C	0.56
		0.54
	S	0.53
		0.53
F140 (control)	C	0.56
		0.56
	S	0.50
		0.52
F25	C	0.49
		0.51
	S	0.48
		0.47
F199	C	0.47
		0.47
	S	0.47

Labelling medium : B

Labelling period : 4 hours

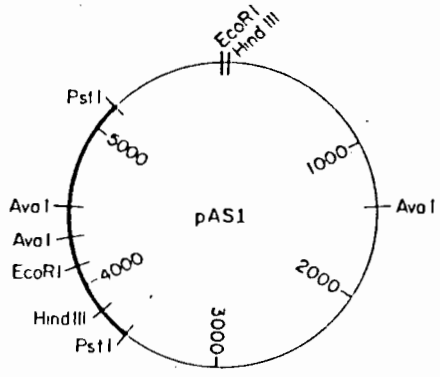
^{14}C-leucine : 0.125 µCi/ml

^{3}H-arginine : 1 µCi/ml

4.5. PREPARATION OF pAS-1 PLASMID DNA

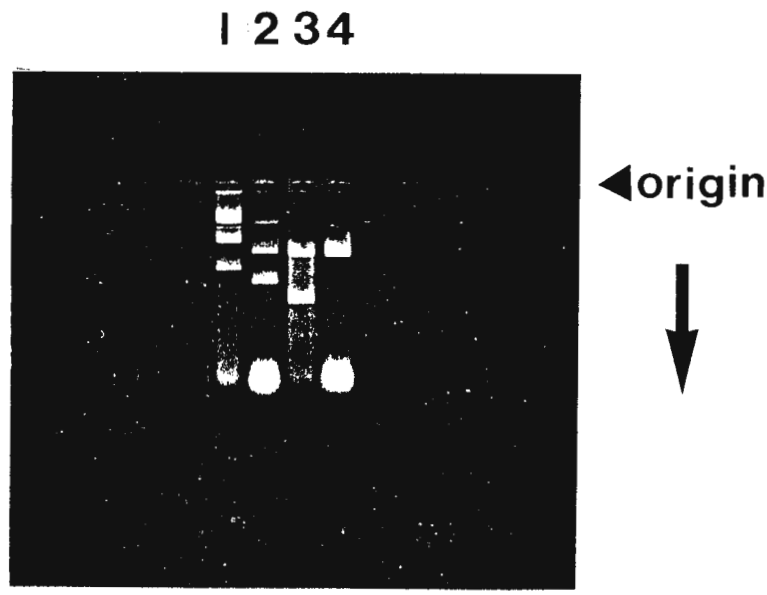
Eighty μg of pAS-1 plasmid DNA was obtained as described in Section 3.3.2. The pAS-1 plasmid consists of a 1.55 kilobase (kb) fragment of cDNA complementary to ASS mRNA inserted at the Pst 1 site of the plasmid pBR322 (Su et al, 1981) (Fig. 4). Consequently, digestion of pAS-1 DNA with Pst 1 should excise the cDNA insert. Visualisation of pAS 1 DNA after electrophoresis on an agarose gel (Fig. 5) with and without digestion with Pst 1 showed that the preparation was largely free of contaminating bacterial DNA. As expected, the cDNA insert was excised by digestion with Pst 1 (Fig. 5).

FIGURE 4



Restriction map of pAS1. The heavy line represents the 1.55 kilobase insert of cDNA complementary to human ASS mRNA. (From Su et al., 1981).

FIGURE 5



Agarose gel electrophoresis of plasmid pAS-1 DNA with and without digestion with Pst 1.

One microgram of pAS 1-DNA was digested with 10 units of Pst 1 for 2 hours. The resulting fragments were electrophoresed on an agarose gel and visualised under UV illumination, after staining with ethidium bromide.

Lane 1: pAS-1 undigested
2: pBR322 undigested
3: PAS-1 digested with Pst 1
4: pBR322 digested with Pst 1

The vertical arrow indicates the direction of electrophoresis.

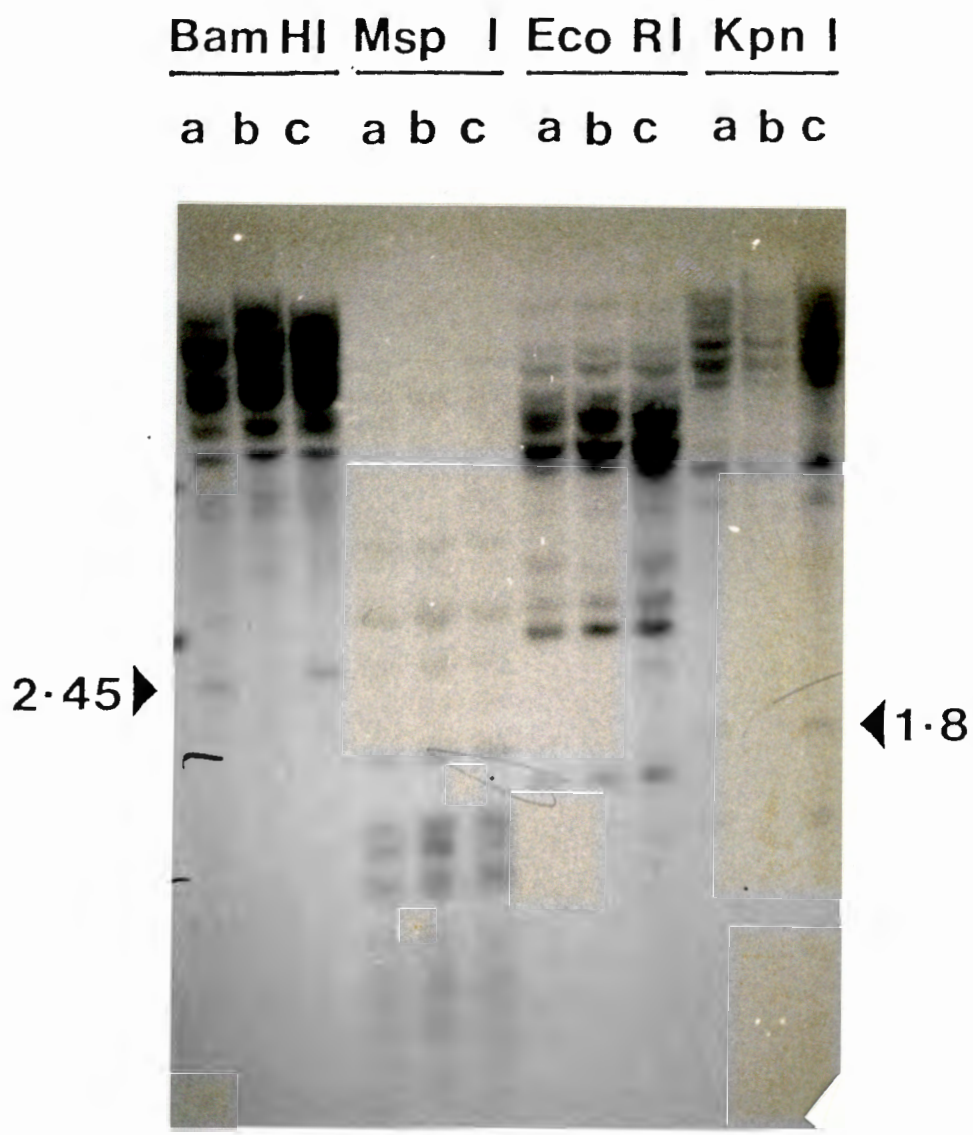
4.6. ANALYSIS OF GENOMIC DNA EXTRACTED FROM CITRULLINEMIC FIBROBLASTS

Approximately 85 x 10^6 F25 (ASS^-) fibroblasts yielded 500 µg of DNA. The restriction endonucleases Bam Hl, Msp 1, EcoRl, Kpn 1, Pst 1 and Hind III were used to digest this DNA in parallel with control DNA samples. After the DNA fragments had been electrophoresed and transferred to nitrocellulose filters they were hybridized with radioactive pAS-1 DNA as described in Sections 3.3.3 to 3.3.7.

The results are shown in Fig. 6.

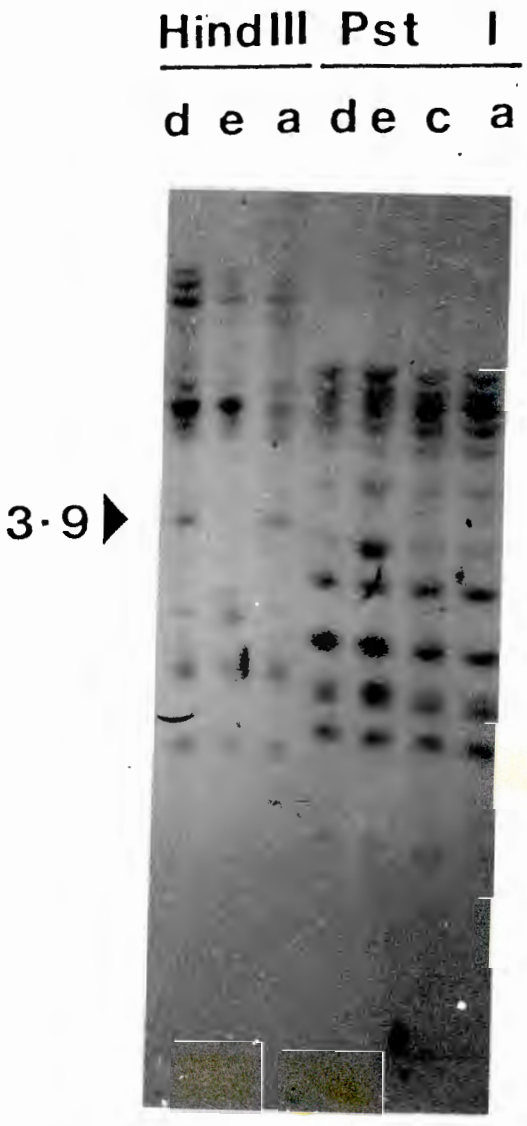
Hybridization of digested genomic DNA from the patient's cells with the pAS-1 probe gave a complex pattern of bands comparable to that previously reported in normal human DNA (Su et al., 1981). The patient, a male, showed the Y-chromosome specific bands (Daiger et al., 1982) as expected. A polymorphism is known to occur among normal individuals at one of the Hind III cutting sites, resulting in fragments of either 3.9 or 2.8 kb in length (Su et al., 1981). The citrullinemic patient was homozygous for the 3.9 kb Hind III fragment. No bands were missing or found in unexpected locations, and the relative intensities of the bands were not different from controls.

FIGURE 6A



Southern blotting analysis of DNA from the citrullinemic patient and control subjects after digestion with restriction enzymes Bam H_1, Msp 1, Eco RI and Kpn 1, and hybridisation to the pAS 1 probe: (a) citrullinemic patient; (b) female control; (c) male control. The arrows indicate the Y chromosomal fragments (1.8 and 2.45 kilobases).

FIGURE 6B



Southern blotting analysis of DNA from the citrullinemic patient and control subjects after digestion with restriction enzymes Hind III and Pst 1 and hybridisation to the pAS 1 probe: (a) citrullinemic patient; (d) and (e) female controls; (c) male control. The arrow indicates the polymorphic 3.9 kilobase Hind III fragment for which the patient was homozygous.

5. DISCUSSION

5.1. CITRULLINE INCORPORATION STUDIES

The citrulline incorporation studies confirm a severe block in the conversion of citrulline to arginine in the two patients. The characteristic amino acid abnormalities in serum and urine identify the missing enzymes as ASS in the F25 cells and ASL in the F199 cells. In both cases, fibroblasts incorporated citrulline at less than 1% of the rate in normal cells. The small amount of radioactivity found in TCA-insoluble material was barely above background and does not necessarily represent true residual enzyme activity. It could equally be the result of (a) trapping of un-incorporated citrulline within the TCA precipitate or (b) contamination of the commercial ^{14}C-citrulline by other ^{14}C compounds. Therefore these results do not exclude the possibility that these patients have a total absence of ASS and ASL activity respectively. Neither the ASS^- nor the ASL^- cell line showed any increase in citrulline incorporation with increasing citrulline concentration, suggesting that neither of the defects is the result of an increased K_m of the enzyme for its substrate.

In the family reported here citrulline incorporation by the parents' fibroblasts was within the normal range. This result is not in agreement with a previous report (Jacoby et al., 1972) which suggested that this method could identify heterozygotes for ASL deficiency, as these cells incorporated citrulline at about half the rate of normal cells.

The results on amniotic fluid cells confirm previous work which shows that amniotic fluid cells have a much wider variation in citrulline-incorporating activity than fibroblasts (Jacoby et al., 1981; Kamoun et al., 1983). Jacoby et al. (1981) established that this is due to a wide variation in ASS activity, while ASL activity is much less variable. High ASS activity is correlated with fibroblast-like cell morphology, and low activity with epitheliod morphology. In the prenatal diagnosis reported here, this was not a problem since a previous sibling (the citrullinemic patient) showed a citrulline incorporation rate well below even the lowest of the control amniotic fluid cell cultures. However, in variants of citrullinemia or argininosuccinic aciduria where the residual enzyme activity is more than 5% of normal, a low result in amniotic fluid cells would have to be interpreted with caution.

Isotope incorporation by whole cells has also been applied to the diagnosis of disorders of propionate, pyruvate, hypoxanthine, ornithine and galactose metabolism (Rozen et al., 1977; Gravel et al., 1975; Shih et al., 1982; Davidson et al., 1984). The rate of isotope incorporation may be related to quantity of protein or DNA, to cell number, or to incorporation of a second isotope as done in the studies reported here. The use of a second label has several advantages which have been discussed previously (Rozen et al., 1977). These include (a) ease and accuracy of quantitation, (b) incorporation of the reference label

occurs only in viable cells, whereas protein, DNA or cell count measurements include dead cells and debris, (c) compensation for variation between cultures in the rate of protein synthesis, if the reference label is an amino acid. The major disadvantage of using a second isotope as a reference is the possibility that variations in the experimental conditions could affect the rate of incorporation of the reference isotope. Finally, in any type of isotope incorporation assay, the composition of the labelling medium, the duration of the labelling period, and the concentration of the isotopes must be standardised, if comparisons between different experiments are to be made.

5.2. DNA ANALYSIS IN CITRULLINEMIA

In the patient with citrullinemia described here no detectable citrulline incorporation could be measured in cultured fibroblasts. This could result from several types of genetic abnormality including major structural alterations of the ASS gene, such as deletions. Deletions have been shown to be the cause of some types of thallassemia (Antonarakis et al., 1982) and growth hormone deficiency (Phillips et al., 1981), and are detectable by analysis of DNA from the patients using the appropriate cloned DNA probe. Most point mutations are likely to be associated with some residual enzyme activity, as has been observed in some cases of citrullinemia (Kennaway et al., 1975).

The pAS-1 probe, which is complementary in sequence to the mRNA for ASS, produces a complex pattern of bands with normal human DNA (Su et al., 1981). This is thought to be due to the presence of several ASS-like sequences in the genome in addition to the ASS gene itself (Beaudet et al., 1982). Distinct Bam Hl and Kpn 1 bands result from sequences located on the Y chromosome (Daiger et al., 1982). A 3.9 kilobase Hind III band is polymorphic due to normal variations in DNA sequence at this site. Beaudet et al. (1982) have assigned this 3.9 kilobase fragment to chromosome 9, and if it is confirmed that it corresponds to the expressed ASS gene, the polymorphism may have value in antenatal diagnosis of citrullinemia.

Su et al. (1982) carried out genomic DNA analysis on DNA from nine citrullinemic patients. All patients studied showed patterns of DNA fragments that were indistinguishable from normal. These authors subsequently showed, using S1 nuclease mapping of mRNA, that several of their patients had abnormalities in the ASS mRNA, and suggested that the most likely defect was an alteration leading to aberrant splicing of the mRNA (Su et al., 1983). The normal pattern of DNA fragments found in the patient reported here indicates that no large deletions are present and suggests that the molecular defect in this case may fall into the same category as the patients reported by Su et al. (1982).

PART B

THE USE OF CULTURED CELLS WITH DEFECTS IN CITRULLINE METABOLISM IN THE STUDY OF INTERCELLULAR COMMUNICATION

6. INTRODUCTION: INTERCELLULAR JUNCTIONAL COMMUNICATION

Many types of eukaryotic cell in organs and tissues as well as in culture form intercellular junctions that allow the direct passage of small molecules between cells. This type of intercellular communication has been the subject of several recent reviews (Loewenstein, 1981; Hooper and Subak-Sharpe, 1981; Hertzberg et al., 1981). In part B of this thesis, a new system for measuring intercellular junctional communication is described and applied to the study of putative inhibitors of junctional intercellular communication.

6.1. THE MEASUREMENT OF JUNCTIONAL INTERCELLULAR COMMUNICATION

Junctional communication was first described by electrophysiologists studying the transmission of action potentials between excitable cells (Furshpan and Potter, 1958; Loewenstein and Kanno, 1964). Certain cells were found to be electrically coupled, in that they were connected by low-resistance junctions. It is now recognised that electrical coupling is a widespread property of many cell types including non-excitable cells, and that the low-resistance junctions are capable of transmitting molecules other than the ions involved in electrical coupling. Intercellular transfer of fluorescein was the first observation of non-electrical transfer via intercellular junctions (Loewenstein and Kanno, 1964; Pappas and Bennett, 1966). The technique of microinjection was subsequently extended to other fluorescent dyes of

different molecular weights, and it was found that dyes of molecular weight less than about 1000 are able to pass between cells, whereas those of greater size than this are not (Loewenstein, 1981).

Other methods for detecting transfer of molecules through intercellular junctions depend on "metabolic co-operation". This term was coined by Subak-Sharpe et al. (1969), who discovered that cells deficient in the enzyme hypoxanthine-guanine phosphoribosyl transferase (HPRT), which are not able to incorporate radiolabelled hypoxanthine by themselves, are able to do so if they are in contact with normal cells. Metabolic co-operation may thus be defined as the alteration of the phenotype of a cell as a result of intercellular junctional communication with another cell. The term metabolic co-operation thus excludes other types of cell interactions which depend on the passage of molecules between cells via the medium, as occurs for example between cells with defects in mucopolysaccharide metabolism (Neufeld, 1974). Several systems for showing metabolic co-operation in cultured cells have been reported. These have been reviewed by Hooper and Subak-Sharpe (1981) and are briefly described below:

(a) $HPRT^-$ cells cultured alone are unable to incorporate radiolabelled hypoxanthine, unlike normal cells. However, when $HPRT^-$ and normal cells are co-cultured, the former cells can be shown by autoradiography to incorporate the label. The original demonstration of metabolic co-operation

in cultured cells was made using this method (Subak-Sharpe et al., 1966; Stoker, 1967; Cox et al., 1974; Cox et al., 1976).

(b) Similar observations apply to cells deficient in adenine phosphoribosyl transferase (APRT) (Bürk et al., 1968).

(c) $HPRT^-$ cells are resistant to the toxic effects of 6-thioguanine since they are unable to convert it to its toxic nucleotide product. Normal cells are killed by 6-thioguanine. When $HPRT^-$ and normal cells are co-cultured both cell types are killed as a result of the passage of the toxic metabolite from the normal cells to the $HPRT^-$ cells via intercellular junctions. This process has been referred to as the "kiss of death" phenomenon (Hooper, 1982), and it has been applied to the study of certain compounds which appear to inhibit intercellular junctional communication (Newbold and Amos, 1981; Warren et al., 1981; Umeda et al., 1980).

(d) In medium containing hypoxanthine, aminopterin and thymidine (HAT medium), survival of cells depends on the presence in the cell of active HPRT enzyme, since de-novo purine synthesis is blocked by aminopterin. Normal cells survive in this medium, but $HPRT^-$ cells are unable to utilise the hypoxanthine and are therefore unable to synthesise purine nucleotides. In co-cultures, the $HPRT^-$ cells are rescued by metabolic co-operation with normal cells, which are able to supply the mutant cells with purine nucleotides. This has been termed the "kiss of life"

phenomenon (Slack et al., 1978) and has also been utilized in the study of putative inhibitors of intercellular communication (Guy et al., 1981).

(e) Donor cells pre-labelled with ^{3}H-uridine transfer radioactive nucleotides to recipient cells via intercellular junctions, and this process can be detected autoradiographically (Pitts and Simms, 1977). This method can also be used with labelled choline derivatives or deoxyglucose (Finbow and Pitts, 1981).

(f) Different cell types differ in their sensitivity to the toxic effect of ouabain, an inhibitor of the Na-K ATPase pump. Sensitive cells accumulate sodium intracellularly and are killed. However ouabain-sensitive cells can be rescued by co-culturing them with ouabain-resistant cells, which are able to relieve the sensitive cells of their sodium load. The mechanism of rescue depends on the passage of sodium between the cells via intercellular junctions (Corsaro and Migeon, 1977).

(g) Lawrence et al. (1978) demonstrated intercellular communication in a most elegant way using co-cultures of myocardial and ovarian granulosa cells. Mouse myocardial cells respond to noradrenalin by an increase in beat frequency. Rat ovarian granulosa cells respond to FSH (Follicle-stimulating hormone) by producing the enzyme plasminogen activator. In co-cultures, noradrenalin elicits plasminogen activator synthesis and FSH elicits the myocardial cell

response, both effects being mediated by intracellular cyclic AMP which is able to cross intercellular junctions.

(h) Chinese hamster DON cells are ASS-deficient and are unable to grow in a citrulline-supplemented, arginine-free medium, unlike normal cells. Cells containing ASS are able to relieve the arginine starvation in the DON cells by transferring arginine and/or argininosuccinate via intercellular junctions (Hooper and Morgan, 1979).

(i) The incorporation of ^{3}H-formate by cells is dependent on the presence of the cofactor tetrahydrofolate. Using co-cultures of folate-starved cells and unstarved cells, it has been shown that tetrahydrofolate passes between the cells via intercellular junctions (Finbow and Pitts, 1981).

(j) A cell type which is both thymidine kinase-deficient and ouabain-resistant is co-cultured with one which contains thymidine kinase and is ouabain-sensitive, and the co-culture is labelled with ^{3}H-thymidine in the presence of ouabain. Neither cell type alone incorporates label into cellular material but in the co-culture, metabolic co-operation allows the ouabain-resistant cell type to rescue the sensitive cell type from the toxic effect of ouabain, and the latter cells can then incorporate the label which can be measured by scintillation counting of TCA-insoluble material (Pitts, 1978).

Direct microinjection techniques offer several advantages in the investigation of intercellular junctional communication.

Firstly, molecules of different size, shape and charge can be used to define in detail the permeability properties of intercellular junctions (Flagg-Newton and Loewenstein, 1979; Flagg-Newton, 1980; Schwartzmann et al., 1981). Secondly, a wide range of cell types can be studied since it is not necessary for the cells to possess biochemical markers such as enzyme deficiencies, nor is it necessary for the cells to be capable of growth in culture. Communication between cells within intact tissues such as vascular endothelium (Larson and Sheridan, 1982) and liver (Meyer et al., 1981) can be investigated by this method.

Thirdly, rapid changes in junctional permeability can be monitored. Using this approach, Fitzgerald et al. (1983) were able to determine the time course of inhibition of intercellular communication due to the tumour-promoter TPA to within a resolution of a few minutes. Fourthly, the effect of perturbing the intracellular milieu can be investigated by injecting appropriate substances into the cell and simultaneously observing dye transfer or electrical coupling. Using these techniques intercellular communication has been reported to be inhibited by increased intracellular calcium concentration (Rose and Loewenstein, 1975; De Mello, 1975; Rose and Loewenstein, 1976; Deleze and Loewenstein, 1976; Flagg-Newton, 1980) and by decreased intracellular pH (Spray et al., 1981; Spray et al., 1984).

Against these advantages must be set the disadvantages of the method, namely the inherent invasiveness of directly

injecting foreign molecules into cells, the technically difficult nature of the procedure, and the fact that the results depend on a subjective interpretation of whether or not dye transfer has occurred and to what extent it has occurred.

With the exception of methods (e) and (i), all the other methods described above require at least one of the two communicating cell types to carry a biochemical marker such as an enzyme deficiency. This limits the range of cell types which can be investigated for intercellular communication; these methods cannot be used to study cells in intact tissues.

Methods (a), (b), (e) and (i) depend on the visualisation of radioactive metabolite transfer by autoradiography. Consequently they require subjective assessment of transfer or time-consuming counting of silver grains. Method (e) is capable of moderately rapid monitoring of the process of establishment of new intercellular junctions: Pederson et al. (1980) were able to show that metabolite transfer occurred within 15 minutes of mixing hepatoma cells using this technique.

Methods (c), (d), (f) (h) and (j) depend on the survival or killing of cells in appropriate selective media. The parameter that is measured (cell death or survival) is removed by many metabolic steps from metabolite transfer,

and it is therefore at best an indirect and semi-quantitative measure of intercellular junctional communication. In addition the processes of cell-killing or rescue require that the cells be co-cultured for an extended period (typically 2 days or more) so that rapid changes in junctional communication cannot be measured (Guy et al., 1981; Tsushimoto et al., 1982; Kurata et al., 1982).

Method (e) has the advantage that mutant cells are not required, and any two cell types in culture can be tested for their ability to communicate. Using this approach Pitts and Burk (1976) demonstrated that some cell types communicated selectively (i.e. only with specific cell types) while other cells were non-selective communicators. Similar conclusions were reached by Fentinan et al. (1976) using the same technique.

6.2. EVIDENCE THAT GAP JUNCTIONS MEDIATE INTERCELLULAR COMMUNICATION

The main lines of evidence implicating the gap junction as the structure responsible for intercellular communication are summarised below. Fuller discussions are given by Bennett (1978), Loewenstein (1979) and Hooper and Subak-Sharpe (1981).

(a) Gap junctions are always present in communicating cell types and are always absent in non-communicating cell types (Gilula et al., 1972).

(b) The gap junction is the only discernible junctional structure in some communicating cells (Gilula et al., 1972; Revel et al., 1971).

(c) Treatments which cause cells to become electrically uncoupled frequently have disruptive effects on gap junctions (Barr et al., 1968; Pappas et al., 1971).

(d) As described in section 6.1 (c), $HPRT^-$ cells are killed when co-cultured with normal cells in the presence of 6-thioguanine, as a result of intercellular junctional communication. A mutation in an $HPRT^-$ cell which resulted in the loss of the capacity for communication would therefore confer resistance to this "kiss of death" phenomenon, and such communication-defective cells would have a selective advantage under these conditions. Using this system, Hooper and co-workers

were able to isolate communication-defective variants (Slack et al., 1978; Hooper and Morgan, 1979) and to show that the loss of the capacity for communication correlated with a simultaneous decrease in the number and size of gap junctions (Hooper and Parry, 1980).

6.3. GAP JUNCTION STRUCTURE

Most current knowledge of gap junction structure comes from studies using the electron microscope. In cross-section the membranes of the two cells in contact are seen to be closely apposed, but are still separated by a 20-30 angstrom gap (from which the junction takes its name) which is penetrated by stain and is therefore contiguous with the extracellular space (Gilula, 1974; Loewenstein, 1979). In freeze-fracture views the junction is seen to be composed of an array of intramembrane particles with a centre-to-centre spacing of 80-100 angstroms (Gilula, 1974). The particles, which have been termed connexons (Goodenough, 1975) are thought to be hexameric proteins, with the six subunits arranged to form a tube containing a central aqueous channel traversing the membrane of each cell. Each connexon in the membrane of one cell is aligned with a connexon in the membrane of the adjacent cell, so that the central channel connects the cytoplasms of the cells and is not leaky to the outside (Zampighi et al, 1980; Unwin and Zampighi, 1980; Baker et al., 1983). The internal diameter of the channel as inferred from the electron micrographic evidence is consistent with the permeability of the junctions to molecules of different sizes: these results imply a diameter of 16 to 20 angstroms for mammalian cells and 20 to 30 angstroms for insect cells (Simpson et al., 1977; Bennett, 1978; Flagg-Newton and Loewenstein, 1979; Finbow and Pitts, 1981; Makowski et al., 1980; Schwartzman et al.,

1981). In practice, this means that the channel is permeable only to molecules of molecular weight less than about 1000.

There is evidence from studies using the sophisticated techniques of Fourier averaging of electron micrographic data (Unwin and Henderson, 1984) that the six subunits can exist in different conformations so that the channel can be *open* or closed (Unwin and Zamphigi, 1980; Wrigley et al., 1984; Unwin and Ennis, 1984). However, the molecular basis for the regulation of junctional permeability remains speculative: Peracchia et al. (1983) propose that the channel-closing mechanism is calmodulin-dependent while de Mello (1984) suggests that reversible phosphorylation of gap junction subunits by a cyclic-AMP-dependent protein kinase may be the regulatory mechanism.

The gap junction is a plaque composed of connexons packed closely in the membrane. A simple model has recently been proposed which explains the maintenance of this large-scale junctional structure in terms of electrostatic forces between connexons and between apposed non-junctional membranes (Peracchia and Bernardini, 1984; Braun et al., 1984). Formation of gap junctions between cells can occur rapidly (within minutes) after cell contact is made: this process does not require the synthesis of new gap junction protein) and is thought to occur by the assembly of junctional poly-peptides pre-existing in the cell membrane (Gilula, 1984).

The half-life of gap junction proteins appears to be of the order of 5 to 10 hours (Fallon and Goodenough, 1981; Yancey et al., 1981).

There appears to be tissue specificity in gap junction structure since the major polypeptides of gap junctions from liver, lens and myocardium lack homology in major regions of their sequences, while the major gap junction protein in any one specific tissue but from widely divergent species is well conserved (Hertzberg and Gilula, 1981; Gros et al., 1983; Larsen, 1983; Revel et al., 1984).

6.4. INHIBITION OF JUNCTIONAL COMMUNICATION BY TUMOR PROMOTERS

Carcinogenesis is now widely perceived as a multistage process (for a review of this subject see Diamond et al., 1980). At least two distinct phases in chemical carcinogenesis, termed "initiation" and "promotion" have been identified in studies on mouse skin, which has been the model system most extensively investigated (Boutwell et al., 1982). A similar process also occurs during tumor induction in other tissues and species (Weinstein et al., 1979). Initiation requires only a single application of the carcinogen, and the initiation event can persist unexpressed for long periods of time. The initiation event is irreversible and is thought to be mutagenic in nature. Initiators are

complete carcinogens, in that they are capable of inducing tumors alone. In contrast, promotion is a slow process requiring repeated exposure to the promoter. Promoters may not be carcinogenic alone: they are capable of enhancing tumor formation only if applied after an initiating stimulus. Promotion is reversible, so that if the order of application of initiator and promoter are reversed, promotion does not occur (Diamond et al., 1980).

The most potent and extensively studied class of tumor promoters are the phorbol esters, for example 12-O-tetradecanoylphorbol-13-acetate (TPA). These compounds induce pleiotropic morphological and biochemical changes in cells, but the relationship of these changes to their tumor-promoting activity remains unclear despite intensive investigation (reviewed in Weinstein et al., 1979). Organochlorine pesticides such as 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT) are also known to possess liver tumor-promoting activity in animals (Peraino et al., 1975; Reuber, 1978).

Both of these groups of compounds (phorbol esters and organochlorine pesticides) have been reported to be inhibitors of junctional communication in several experimental systems (Yotti et al., 1979; Fitzgerald and Murray, 1980; Umeda et al., 1980; Enomoto et al., 1981; Guy et al., 1981; Newbold and Amos, 1981; Warren et al., 1981; Williams et al., 1981; Kurata et al., 1982; Tsushimoto et al., 1982;

Friedman and Steinberg, 1982; Mosser and Bols, 1982; Trosko et al., 1982; Fitzgerald et al., 1983; Tsushimoto et al., 1983; Walder and Lutzelschwab, 1984). Inhibition of junctional communication is one of the earliest cellular effects of TPA, being detectable in cultured cells within 10 minutes of exposure (Fitzgerald et al., 1983).

The correlation between tumor-promoting activity and inhibition of intercellular communication has strengthened the hypothesis (Loewenstein, 1979) that normal growth control requires intercellular junctional communication. According to this hypothesis, cellular growth control may be mediated by growth-regulating molecules which are transmitted between cells via gap junctions. Compounds which inhibit junctional communication would allow cells to escape these normal growth-regulating signals and as a result enhance the formation of tumors from initiated cells (Trosko et al., 1982).

It has been suggested that inhibition of intercellular communication may be a general property of tumor promoters, and that this property might be a means of identifying compounds with tumor-promoting activity (Trosko et al., 1982; Kurata et al., 1982).

Retinoic acid (RA) and other retinoids have been shown to inhibit tumor promotion by phorbol esters (Verma et al., 1979; Sporn et al., 1976; Slaga et al., 1980; Lotan, 1980) and to prevent or modify several of the effects of tumor promoters

on cells (Verma and Boutwell, 1977; Kensler and Mueller, 1978). The reports that retinoids themselves also have the property of inhibiting junctional communication (Pitts et al., 1982; Walder and Lutzelschwab, 1984) would therefore appear to be in conflict with the general hypothesis that inhibition of junctional communication is causally related to tumor promotion. In view of this apparent discrepancy it would be valuable to confirm that both RA and TPA were inhibitors of junctional communication in the same system, and to test whether any antagonism could be demonstrated between these compounds regarding their effects on intercellular communication. In addition it would be of interest to determine whether fluocinolone acetonide (FA), which has potent anti-tumor-promoting activity but is thought to act at a different stage in tumor promotion from RA (Slaga et al., 1980), would antagonise the inhibitory effect of TPA on junctional communication.

6.5. CO-CULTURES OF ASS$^-$ AND ASL$^-$ CELLS

The results described in Sections 4.1 to 4.4 were obtained in an attempt to evaluate optimal double-labelling conditions for diagnostic purposes. During the course of this work we observed that although fibroblasts deficient in ASS or ASL are unable to incorporate ^{14}C-citrulline into protein (Sections 4.1 and 4.2), when these two cell types are mixed together and cultured, ^{14}C-citrulline is incorporated by the co-culture at rates which approach those in normal cells. The data

presented in the following sections show that this surprising phenomenon is the result of metabolic co-operation, i.e. the exchange of metabolites via intercellular junctions between the two cell types. The effects of changes in extracellular calcium, magnesium and pH on metabolic co-operation are reported. The organochlorine pesticide DDT (1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane) is shown to be an inhibitor of intercellular junctional communication in this system.

Chinese hamster V79 cells are deficient in ASS activity (Gonzalez-Noreiga et al., 1980) and in Section 8.14 it is shown that these cells can be used instead of ASS^- human fibroblasts in co-cultures for measuring intercellular junctional communication. This extends the usefulness of the system, since V79/ASL^- co-cultures are more sensitive to communication inhibition by the phorbol ester TPA than co-cultures of ASS^- and ASL^- human fibroblasts, as is shown in Section 8.15.

Inhibition of junctional communication by retinoids and the interaction of retinoic acid and TPA are examined in Sections 8.16 and 8.17.

7. MATERIALS AND METHODS

The origin of the human cells and methods of culture are described in Section 3.1. Chinese hamster V79 cells (subclone 379A) were from Flow Laboratories, Scotland.

7.1. PREPARATION OF CO-CULTURES

Confluent monolayers of ASS^- (F25) and ASL^- (F199) fibroblasts were trypsinised to detach the cells. The cell suspensions were agitated by pipetting to disrupt clumps of cells and an appropriate volume of Eagles Basal Medium with 10% or 15% foetal calf serum and antibiotics was added. Except where stated, F25 and F199 cells were mixed in equal ratio. Where the two cell types were mixed in different ratios, aliquots of each suspension were counted in quadruplicate using a Coulter model ZF counter, and the volumes of the suspensions to be mixed were adjusted accordingly to give the desired ratio. The mixed suspensions were then pipetted into 60 mm dishes at a density of 10^6 cells per dish, except where stated. Radioisotope labelling experiments were performed on the following day, except where otherwise stated. In later experiments, 6-well plates (well diameter 35 mm, Nunc) were used. These co-cultures will be referred to as ASS^-/ASL^- co-cultures.

For co-cultures between ASL^- human fibroblasts and V79 cells, the former were plated into 60 mm petri dishes (10^6 cells per dish) or wells (diameter 35 mm, 5×10^5 cells per well)

of 6-well plates. After allowing 2 hours for the human cells to attach, an equal number of V79 cells were added to each dish. This procedure was adopted as it was found that if the human fibroblasts and V79 cells were mixed and plated together, the V79 cells would preferentially attach to the substratum and inhibit the attachment of the human cells. These will be referred to as V79/ASL$^-$ co-cultures.

Incorporation of isotopes and extraction of TCA-insoluble material were as described in Section 3.2. Labelling conditions are given with the results of each experiment, except in Sections 8.14 to 8.17. In these sections all experiments were performed using medium Z for labelling, and both ^{14}C-citrulline and ^{3}H-phenylalanine were used at activities between 0.2 and 0.5 µCi/ml.

7.2. AUTORADIOGRAPHY OF CELL CULTURES

Cell suspensions of ASS^- and ASL^- cells and mixtures of these suspensions were plated into chambers of 8-chambered tissue culture slides (Lab-Tek Products, Naperville, Illinois). After two hours the medium was replaced with BME (serum free) containing 2 µCi/ml of ^{14}C-citrulline and incubated overnight. The cells were then washed twice with serum-free BME and incubated in serum-free BME with 1 mM unlabelled citrulline for two hours. After washing the cells with Dulbecco's phosphate-buffered saline, the slides were placed in 10% formaldehyde in normal saline for 15 min and washed in running water for 30 minutes. After air drying, the slides were coated with Kodak NTB-2 emulsion, exposed at -70°C for two weeks, developed, and stained lightly with methylene blue.

7.3. PREPARATION OF LABELLING MEDIA WITH DEFINED LOW CALCIUM CONCENTRATION

Where the desired concentration of free calcium in the medium was 30 µmol/ℓ or less, calcium-EGTA buffers were used as described by Vianna (1975), with a total EGTA concentration of 1 mM and calcium chloride added in varying amounts. The pH of the solutions were adjusted to 7.4 after adding the calcium chloride, as the addition of calcium to EGTA-containing solutions causes the release of protons from EGTA.

7.4. ADDITION OF DDT TO MEDIA

DDT (1,1,1-trichloro-2,2-bis(p-chlorophenyl)-ethane) was obtained from Sigma Chemical Co., and dissolved in ethanol at 100 times the desired final concentration in the medium. Thus the final ethanol concentration was 1% (v/v). An equal amount of ethanol was added to control cultures without DDT. At concentrations of DDT above 10 µg/ml, the solutions became opalescent, indicating incomplete dissolution of the DDT; hence the indicated concentrations must be considered nominal. Preliminary experiments showed that 1% ethanol did not affect isotope incorporation or metabolic co-operation significantly.

12-O-Tetradecanoylphorbol-13-acetate (TPA) and fluocinolone acetonide (FA) were obtained from Sigma, dissolved in ethanol and stored at -20°C. Retinoic acid (all trans), retinal, retinol and retinyl acetate were obtained from Sigma, dissolved in dimethyl sulphoxide (DMSO) and stored at -20°C in the dark. All the above compounds were added to the media to give a final ethanol or DMSO concentration of 0.5% (v/v). All manipulations with retinoids were performed in subdued light.

8. RESULTS

8.1. METABOLIC CO-OPERATION BETWEEN ASS$^-$ AND ASL$^-$ CELLS

As shown in Sections 4.1 and 4.2, ASS$^-$ and ASL$^-$ show very low levels of ^{14}C-citrulline incorporation when cultured separately. However, ^{14}C-citrulline incorporation increased markedly when these two cell types were grown in mixed culture. The results of a typical co-culture experiment are shown in Table 4. In seven experiments performed on different occasions, ^{14}C-citrulline incorporation by co-cultures was an average of 130 fold higher than the levels in the ASS$^-$ and ASL$^-$ cells separately (Fig. 7). Under the conditions of these experiments, citrulline incorporation in co-cultures was equal to that in normal fibroblasts (Fig. 7).

8.2. VARYING PROPORTIONS OF ASS$^-$ AND ASL$^-$ CELLS

By mixing the two cell types in varying proportions and plotting the ^{14}C DPM/^{3}H DPM ratio against percentage of cell type, a curve was obtained which showed that complementation reached a maximum with a mixture of 25% ASS$^-$ and 75% ASL$^-$ cells, under conditions where no additional unlabelled citrulline was added (Table 5 and Fig. 8A).

The curve of metabolic co-operation as a function of varying proportions of the two cell types (Fig. 8A) was skewed towards the right, indicating that the admixture of as little as 4% ASS$^-$ cells with a culture of ASL$^-$ cells was capable of

TABLE 4

RESULTS OF A TYPICAL CO-CULTURE EXPERIMENT

CELL LINE	^{3}H DPM	^{14}C DPM	$\frac{^{14}C \text{ DPM}}{^{3}H \text{ DPM}} \times 100$
F 140 (control)	52811	4992	9.45
	59733	5520	9.24
F 25	44064	31	0.07
	37819	28	0.07
F 199	21122	33	0.15
	23804	25	0.10
F25/F199 CO-CULTURE	32213	3257	10.1
	28876	3075	10.7

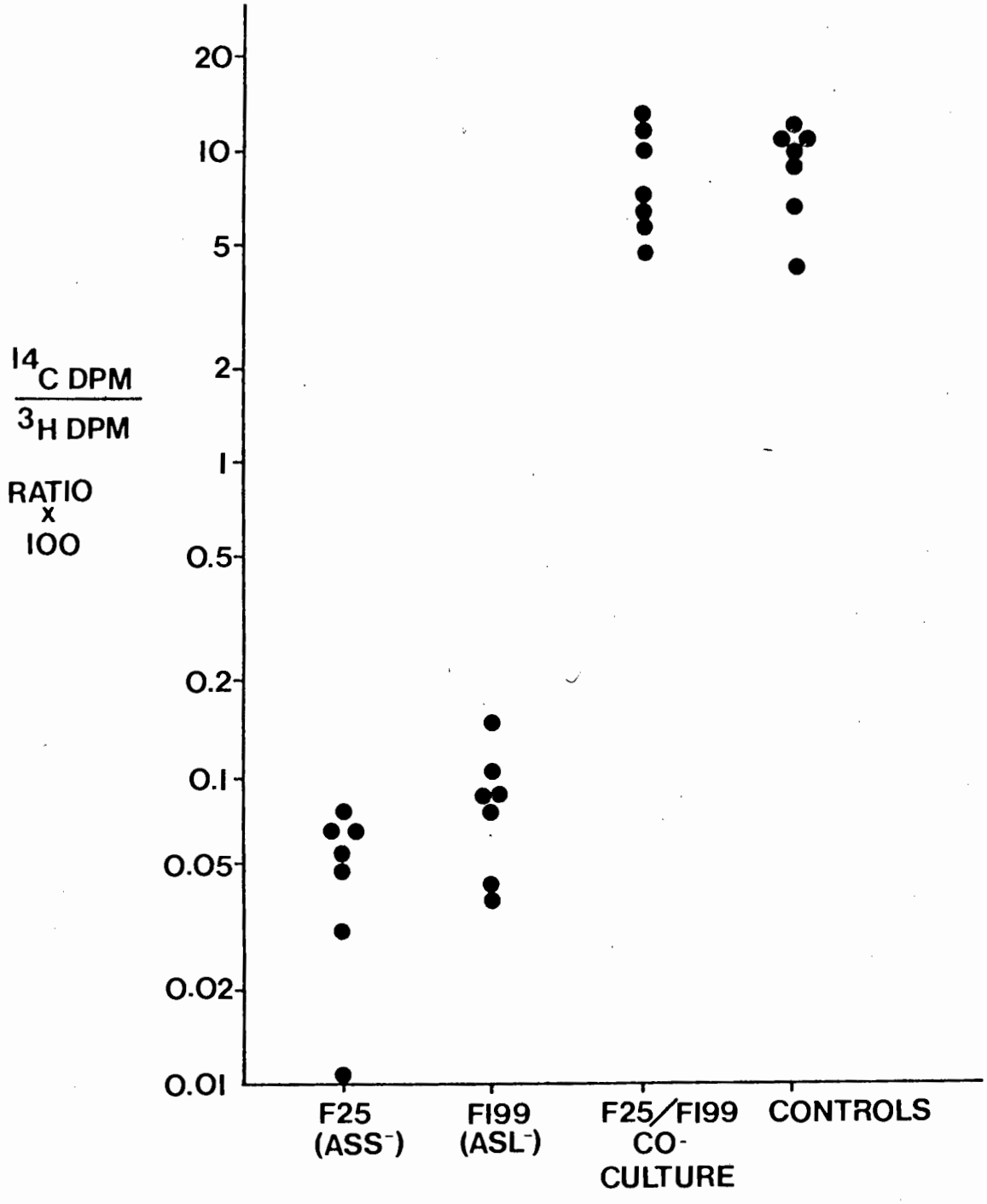
Labelling medium : B

Labelling period : 5 hours

^{14}C-citrulline : 0.5 µCi/ml

^{3}H-leucine : 0.5 µCi/ml

FIGURE 7



Results of 7 co-culture experiments

Labelling medium : B
Labelling period : 5 hours
^{14}C-citrulline : 0.5 µCi/ml
^{3}H-leucine : 0.5 µCi/ml

TABLE 5

METABOLIC CO-OPERATION IN CO-CULTURES OF DIFFERING PROPORTIONS OF ASS-DEFICIENT AND ASL-DEFICIENT HUMAN FIBROBLASTS

% of Cells		^{3}H-DPM	^{14}C-DPM	$\frac{^{14}\text{C-DPM}}{^{3}\text{H-DPM}}$ RATIO
F25 (ASS-deficient)	F199 (ASL-deficient)			
100	0	30931	17	0.0006
98	2	31116	240	0.0077
96	4	31444	501	0.0159
94	6	27220	608	0.0223
90	10	36664	1253	0.0342
75	25	38221	2825	0.0739
50	50	28889	3308	0.1145
25	75	34585	4356	0.1260
10	90	32871	3656	0.1112
6	94	36620	3024	0.0826
4	96	32639	2253	0.0690
2	98	35087	1346	0.0384
0	100	36170	25	0.0007

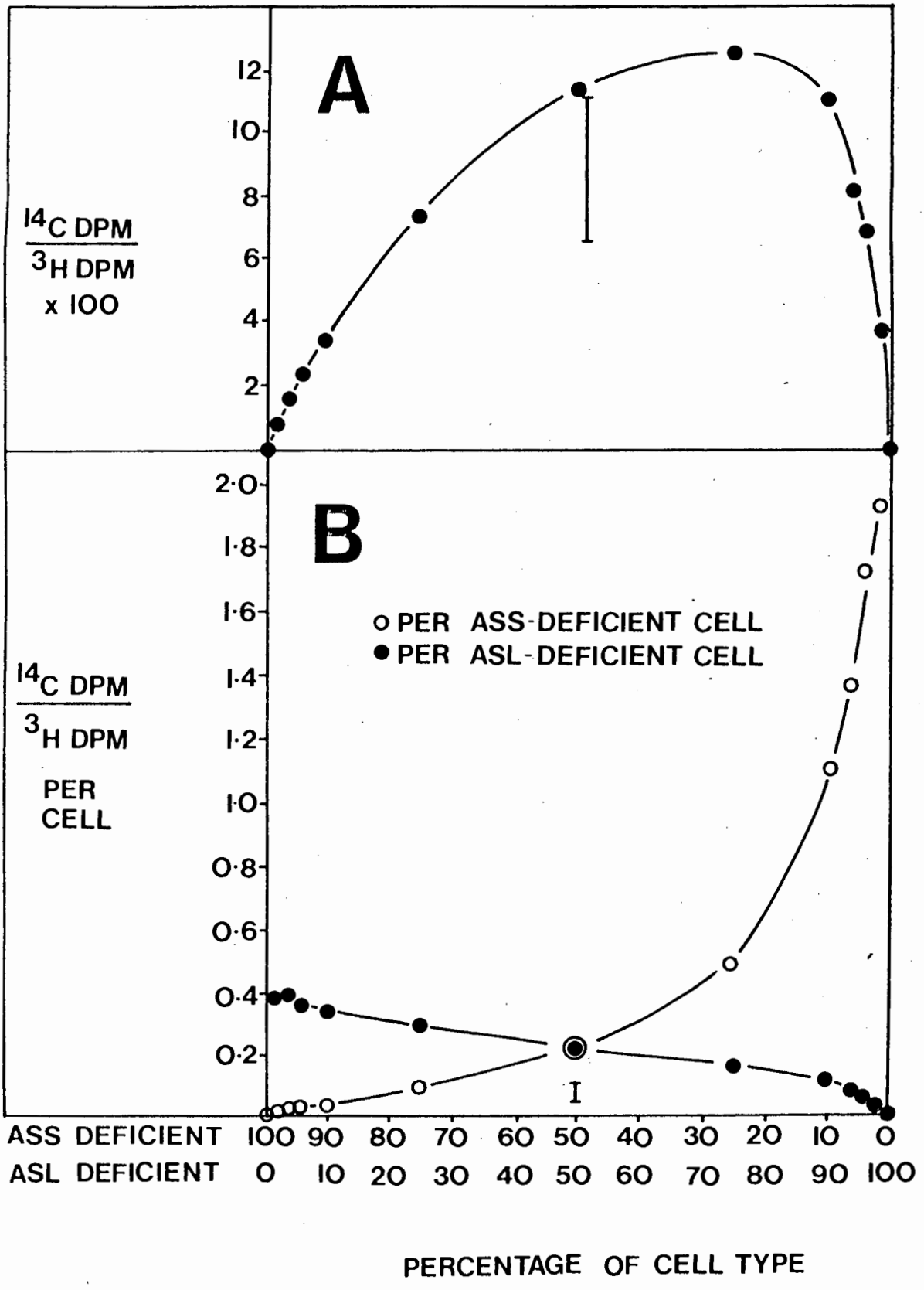
Labelling medium : B

Labelling period : 5 hours

^{14}C-citrulline : 0.5 µCi/ml

^{3}H-leucine : 0.5 µCi/ml

FIGURE 8


A
B
^{14}C DPM / ^{3}H DPM x 100
^{14}C DPM / ^{3}H DPM PER CELL
o PER ASS-DEFICIENT CELL
● PER ASL-DEFICIENT CELL
I
ASS DEFICIENT 100 90 80 70 60 50 40 30 20 10 0
ASL DEFICIENT 0 10 20 30 40 50 60 70 80 90 100
PERCENTAGE OF CELL TYPE

LEGEND TO FIGURE 8

A. Graphical representation of data shown in Table 2: Metabolic co-operation between ASS-deficient and ASL-deficient fibroblasts as a function of the proportion of each cell type in the co-culture. Total citrulline concentration : 0.01 mM.

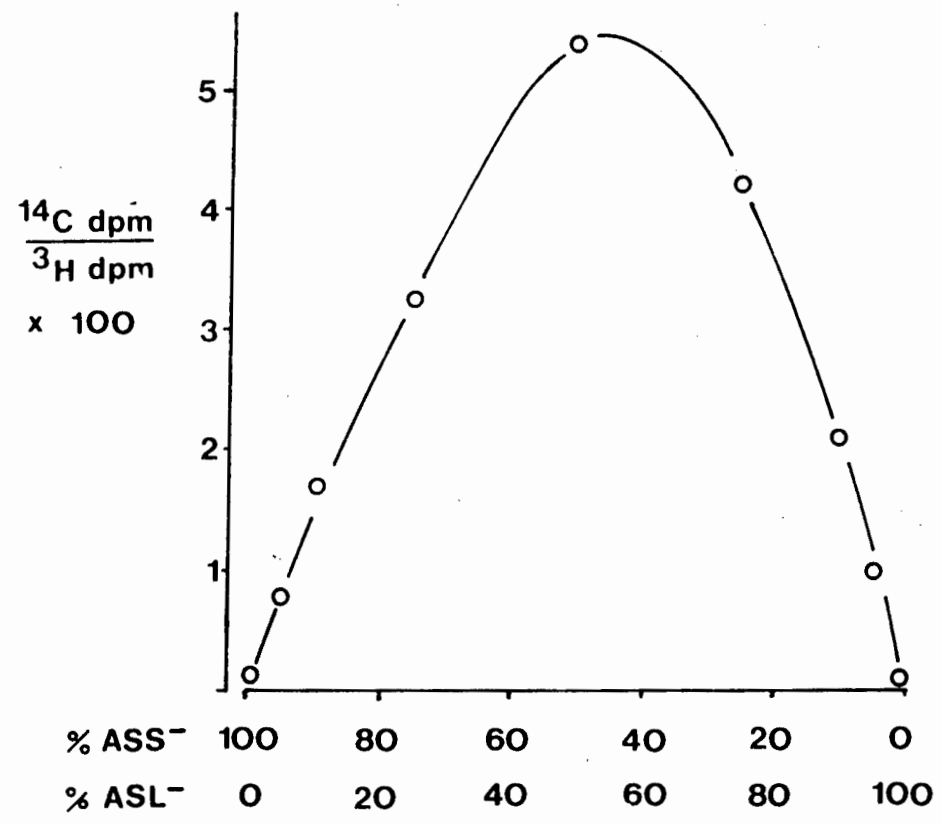
B. Mean citrulline-derived relative flux per cell as a function of the percentage (p) of ASL-deficient cells in the co-culture. Mean flux per ASS-deficient cell (O) is calculated as $\frac{^{14}C\ DPM}{^{3}H\ DPM} \times \frac{100}{100-p}$. Mean flux per ASL-deficient cell (●) is calculated as $\frac{^{14}C\ DPM}{^{3}H\ DPM} \times \frac{100}{p}$

The bars in the centre of the figures represent the mean $\pm$ SD of the values found in normal fibroblasts (the data for normal fibroblasts is shown in Fig. 7).

restoring ^{14}C-citrulline incorporation relative to ^{3}H-Leucine by the whole culture to half maximal levels. The assay was sensitive enough to detect metabolic co-operation in co-cultures consisting of 98% of one cell type and 2% of the other cell type. The data of Table 5 could also be expressed as mean flux of citrulline derived label relative to leucine derived label per cell of a given type (Fig. 8B). The resulting curves showed that when ASS$^-$ cells constituted 2% of the co-culture, the relative citrulline derived flux per ASS-deficient cell was 20 fold greater than in normal cells under the same conditions. When ASL$^-$ cells constituted 2% of the co-culture the relative citrulline derived flux per ASL$^-$ cell was 4-fold greater than the flux per normal cell under the same conditions.

When unlabelled citrulline was added to give a final citrulline concentration of 0.6 mM the curve of metabolic co-operation plotted against proportion of cell type had a more symmetrical shape (Fig. 9). As was shown in Section 4.2, at this concentration the citrulline incorporation pathway is operating near its maximal flux in normal cells.

FIGURE 9



Metabolic co-operation in relation to proportion of cell types in the co-culture. Unlabelled citrulline was added to give a final citrulline concentration of 0.6 mM

Labelling medium : B

Labelling period : 5 hours

^{14}C-citrulline : 1 µCi/ml

^{3}H-leucine : 0.5 µCi/ml

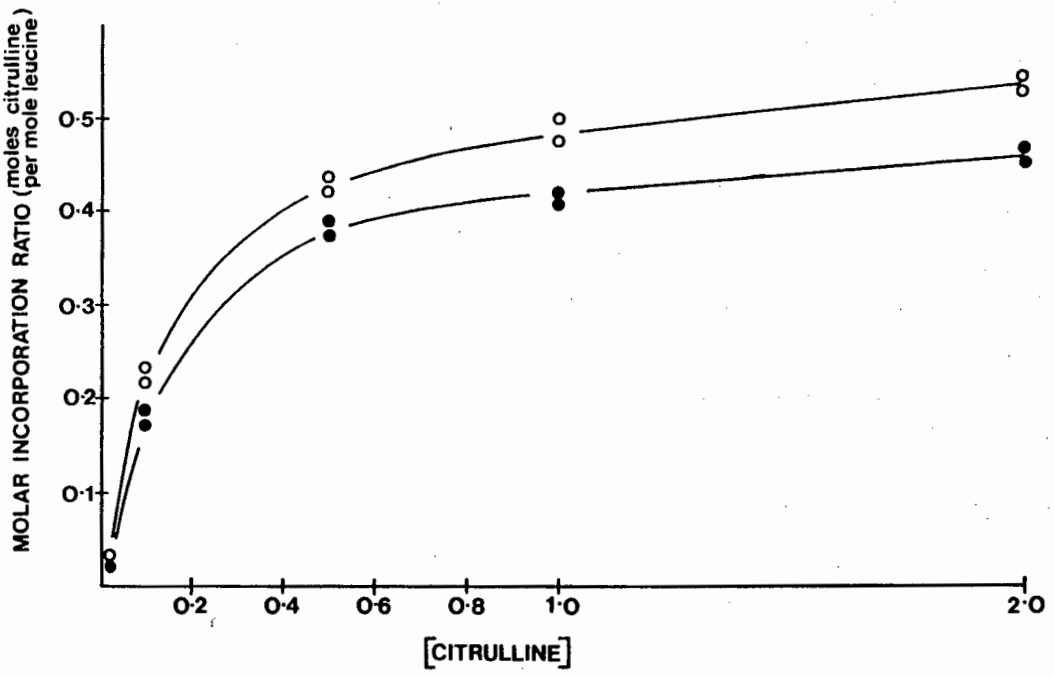
8.3. CITRULLINE INCORPORATION BY CO-CULTURES IN RELATION TO EXTERNAL CITRULLINE CONCENTRATION

The maximal rate of citrulline incorporation was determined for a normal cell line and for a co-culture of 25% ASS^- and 75% ASL^- cells by adding increasing amounts of unlabelled citrulline to the labelling medium. The maximal rate of citrulline incorporation in the co-culture was similar to that in a normal cell line (Fig. 10). At a citrulline concentration of 2 mM the rate of citrulline incorporation was 2.8×10^5 molecules of citrulline per ASS-deficient cell per second. Both the co-culture and the normal cell line showed saturable kinetics, with half-maximal citrulline incorporation at a citrulline concentration of about 0.2 mM.

8.4. TIME OF ESTABLISHMENT OF METABOLIC CO-OPERATION

Serial measurements at varying times after mixing the cell suspensions (Fig. 11) showed that metabolic co-operation was well established after one hour of co-culture and was maximal by two to three hours.

FIGURE 10



Incorporation of citrulline as a function of citrulline concentration in the medium, expressed as moles of citrulline per mole of leucine incorporated into TCA-precipitable material. O - O, normal fibroblasts (F140); ● - ●, co-culture consisting of 25% ASS-deficient (F25) cells and 75% ASL-deficient (F199) cells.

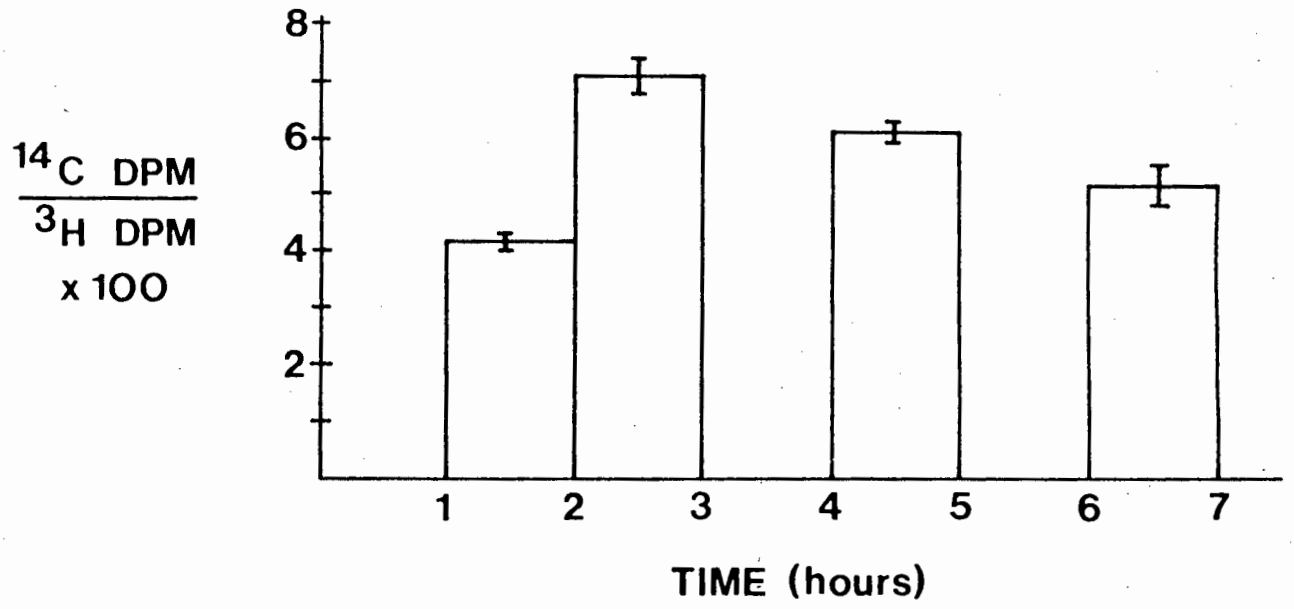
Labelling medium : A

Labelling period : 4 hours

^{14}C-citrulline : 0.25 µCi/ml

^{3}H-leucine : 1 µCi/ml

FIGURE 11



Time course of establishment of metabolic co-operation. The ends of the bars represent the values obtained in duplicate dishes.

Labelling medium : B

Labelling period : 1 hour

^{14}C-citrulline : 2 µCi/ml

^{3}H-leucine : 2 µCi/ml

8.5. DEPENDENCE OF METABOLIC CO-OPERATION ON CELL DENSITY

A suspension of ASS^- and ASL^- cells in equal numbers was plated onto 5 tissue culture dishes of different sizes, using the same volume of cell suspension for each dish, so that in the smallest dishes the cells were confluent and in the largest dishes they were sparse, and therefore the only variable was the area on which the cells were plated. Metabolic co-operation as measured by the ^{14}C DPM/^{3}H DPM ratio increased with increasing cell density (Fig. 12).

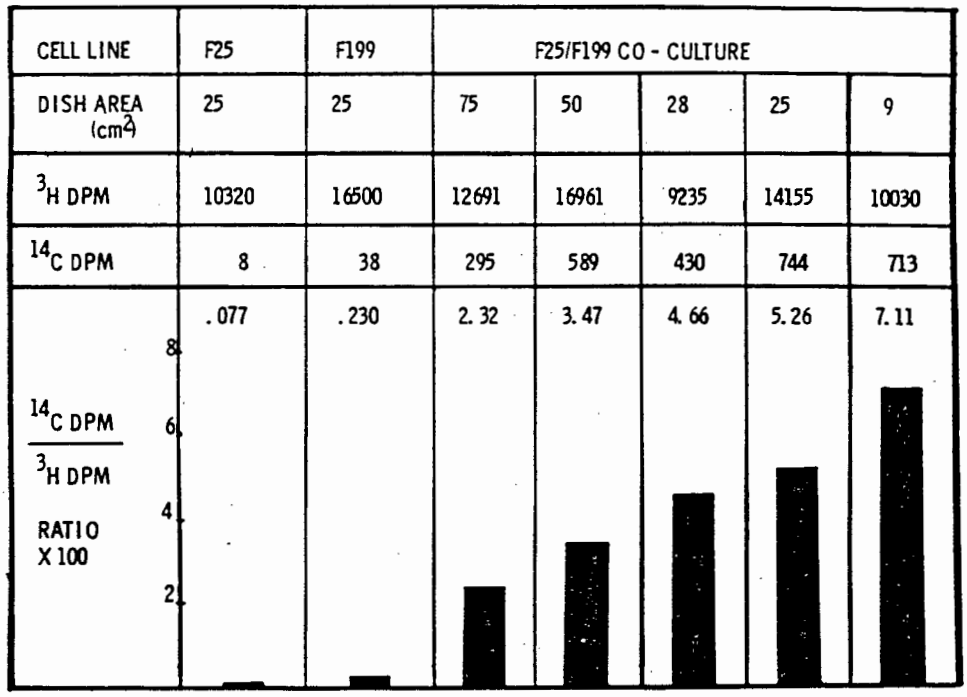
8.6. MEDIA EXCHANGE EXPERIMENTS

To test whether metabolic co-operation was due to release of a metabolic intermediate into the medium, two essentially similar types of experiment were performed. In the first type of experiment duplicate cultures of ASS-deficient and ASL-deficient fibroblasts were incubated in labelling medium, and after 3 hours the medium from the ASS^- cells was decanted and exchanged with that from the ASL^- cells. The cells were incubated for a further 3 hours and the results were compared with cultures in which the media were not exchanged, and with co-cultures incubated for 6 hours. Citrulline incorporation did not increase significantly when the medium was exchanged (Table 6).

In the second type of experiment, four 60 mm dishes were divided into two compartments with partitions, sealed with

FIGURE 12

CELL LINE	F25	F199	F25/F199 CO - CULTURE				
DISH AREA (cm^2)	25	25	75	50	28	25	9
^3H DPM	10320	16500	12691	16961	9235	14155	10030
^{14}C DPM	8	38	295	589	430	744	713
$\frac{^{14}C\ DPM}{^3H\ DPM}$ RATIO X 100	.077	.230	2.32	3.47	4.66	5.26	7.11



Dependence of metabolic co-operation on cell density. A suspension of approximately equal numbers of ASS^- and ASL^- cells was plated into 5 dishes of different sizes, the same volume of suspension being used for each dish.

Labelling medium : B

Labelling period : 4 hours

^{14}C-citrulline : 0.5 µCi/ml

^3H-leucine : 0.5 µCi/ml

TABLE 6

EXCHANGE OF LABELLING MEDIA

Midway through the labelling period of 6 hours the media of the cultures marked (*) were exchanged. The results of a second identical experiment are shown in brackets.

CELL LINE	^{3}H DPM	^{14}C DPM	$\frac{^{14}C \text{ DPM}}{^{3}H \text{ DPM}} \times 100$
F25 (ASS^{-})	23676	7	0.03 (0.06)
F199 (ASL^{-})	22820	8	0.04 (0.01)
F25/F199 CO-CULTURE	24941	708	2.84 (2.37)
F25 *	39703	22	0.06 (0.08)
F199 *	31134	22	0.07 (0.01)
F9 (CONTROL)	20583	426	2.07 (2.07)
F8 (CONTROL)	17907	437	2.44 (5.70)
F32 (CONTROL)	29794	803	2.70 (5.10)

Labelling medium : B

Labelling period : 6 hours

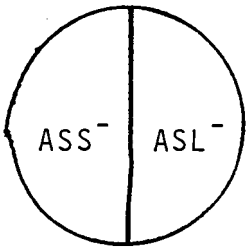
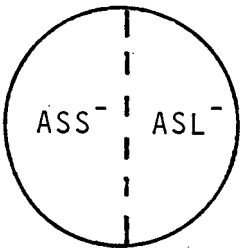
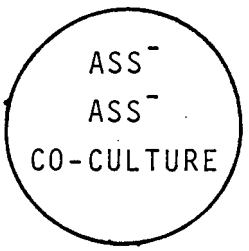
^{14}C-citrulline : 0.25 µCi/ml

^{3}H-leucine : 0.5 µCi/ml

agarose. ASS^- and ASL^- cells were seeded into adjacent halves of the dishes at confluent density. Co-culture dishes were identical except that there were no partitions and they were seeded with a mixed suspension (co-culture) of ASS^- and ASL^- cells. On the following day the partitions were removed from two of the dishes, and all dishes were incubated with 2 ml of labelling medium, with gentle shaking (60 rpm) on a rotary platform. In the dishes with the partitions removed, therefore, the two cell types, although physically separated, were bathed in the same medium. No increase in ^{14}C DPM/^{3}H DPM ratio was seen in the dishes with the partitions removed (Table 7).

The results of both types of experiment indicate that metabolic co-operation in this system is not due to the release of a metabolic intermediate or other factor into the medium.

TABLE 7

	^{3}H DPM	^{14}C DPM	$\frac{^{14}C \text{ DPM}}{^{3}H \text{ DPM}} \times 100$
 ASS⁻ AND ASL⁻ CELLS SEPARATE, MEDIA SEPARATE	35183	32	0.09
	24658	17	0.07
 ASS⁻ AND ASL⁻ CELLS SEPARATE, COMMON MEDIUM	35736	24	0.07
	35334	49	0.14
 CO-CULTURE IN COMMON MEDIUM	42004	4244	10.1
	35053	3599	10.3

Absence of metabolic co-operation when ASS⁻ and ASL⁻ cells are physically separated and incubated in duplicate in the same labelling medium.

Labelling medium : B

Labelling period : 5 hours

^{14}C-citrulline : 0.5 µCi/mol

^{3}H-leucine : 0.5 µCi/ml

8.7. ADDITION OF UNLABELLED ARGININOSUCCINATE

The addition of unlabelled argininosuccinate to the medium (1 mM) caused a small decrease in ^{14}C-citrulline incorporation by co-cultures. A decrease of similar magnitude was seen in a normal cell line (Table 8). In this experiment the initial external citrulline concentration was 0.01 mM. Therefore, even if all of this were converted (by the ASL$^-$ cells) into ^{14}C-argininosuccinate and released into the medium, it would be diluted 100 fold by the 1 mM unlabelled argininosuccinate. In practice, less than 1% of the ^{14}C-citrulline present in the labelling medium is incorporated in the course of labelling experiments of this type, and the actual dilution of ^{14}C-argininosuccinate released into the medium would therefore be closer to 10 000 fold. These results confirm that the complementation between ASS$^-$ and ASL$^-$ cells is not due to release of ^{14}C-argininosuccinate into the medium by ASL$^-$ cells and uptake by ASS$^-$ cells.

8.8. ABSENCE OF METABOLIC CO-OPERATION BETWEEN ASS$^-$ FIBROBLASTS AND ASL$^-$ LYMPHOBLASTS

The ASL-deficient lymphoblastoid cell line L199 was derived from the same patient as the F199 fibroblast line (Section 3.1.2). When L199 cells were co-cultured with F25 (ASS$^-$) fibroblasts, metabolic co-operation did not occur (Table 9). The lymphoblasts did not adhere to the fibroblast monolayer.

TABLE 8

EFFECT OF ADDING UNLABELLED ARGININOSUCCINATE TO THE LABELLING MEDIUM ON CITRULLINE INCORPORATION IN CO-CULTURES OF ASS^- AND ASL^- CELLS AND ON NORMAL FIBROBLASTS

CELL LINE	ARGININO-SUCCINATE (mM)	^3H DPM	^{14}C DPM	$\frac{^{14}C \text{ DPM}}{^3H \text{ DPM}} \times 100$
FG	0	50836	5292	10.2
(CONTROL)		57721	5790	10.0
	1	52993	4154	7.84
		14184	1069	7.54
ASS^-/ASL^-	0	63241	5851	9.25
		53236	5078	9.53
CO-CULTURE	1	41601	3533	8.49
		48141	3990	8.29

Labelling medium : B

Labelling period : 5 hours

^{14}C-citrulline : 0.5 µCi/ml

^3H-leucine : 0.5 µCi/ml

TABLE 9

ABSENCE OF METABOLIC CO-OPERATION BETWEEN ASS-DEFICIENT FIBROBLASTS (F25) AND ASL-DEFICIENT LYMPHOBLASTS (L199)

10^6 Epstein-Barr Virus-transformed ASL-deficient lymphoblasts (L199) were co-cultured with 6 x 10^5 ASS-deficient fibroblasts (F25), and ^{14}C-citrulline incorporation relative to ^{3}H-leucine was compared with that in a co-culture of 6 x 10^5 F25 cells and the same number of ASL-deficient fibroblasts (F199).

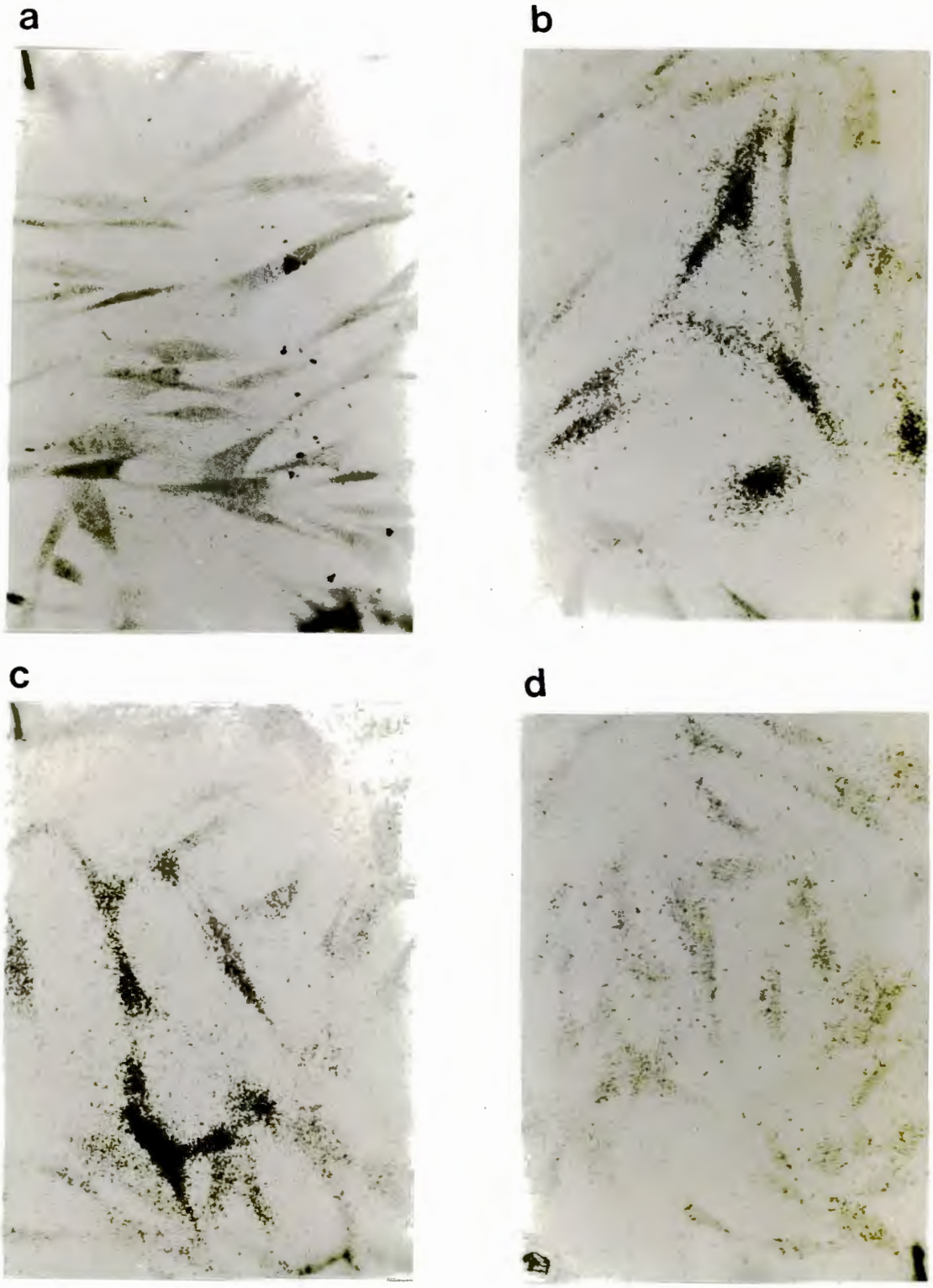
CELL LINE	^{3}H DPM	^{14}C DPM	$\frac{^{14}C\ DPM}{^{3}H\ DPM} \times 100$
F25	40900	23	0.06
	41910	25	0.06
F25/F199	34797	3571	10.3
CO-CULTURE	30146	3242	10.7
F199	20381	19	0.09
	20336	20	0.10
F25/L199	47190	27	0.06
	46750	26	0.06
L199	25057	15	0.06
	20787	14	0.07

Labelling medium : B
Labelling period : 5 hours
^{14}C-citrulline : 0.5 µCi/ml
^{3}H-leucine : 0.5 µCi/ml

8.9. AUTORADIOGRAPHY OF CO-CULTURES AFTER LABELLING WITH ^{14}C-CITRULLINE

In order to visualise the process of metabolic co-operation, co-cultures consisting of different proportions of ASS^- and ASL^- fibroblasts were labelled with ^{14}C-citrulline, chased with unlabelled citrulline, fixed, and autoradiographed. ASS^- cells were not distinguishable from ASL^- cells morphologically. When labelled separately in ^{14}C-citrulline, both ASS^- and ASL^- fibroblasts showed only negligible labelling above background, which was present equally in all cells (Figs. 13A and 13D). When 97% ASS^- cells were co-cultured with 3% ASL^- cells, most cells showed no difference in labelling from the ASS^- culture, while isolated groups of 5 to 15 adjacent cells were more heavily labelled (Fig. 13B). Within these groups the central few cells were equally heavily labelled, while other cells on the edge of the group were less heavily labelled. When 3% ASS^- cells were co-cultured with 97% ASL^- cells, groups of 5-15 heavily labelled cells were again seen (Fig. 13C). In these latter groups there was usually a single cell at the centre of the group which was very heavily labelled, with surrounding adjacent cells less heavily labelled. These central cells were present at a frequency of approximately 3% and are presumed to be the ASS^- cells.

FIGURE 13



Autoradiography of co-cultures after incubation with ^{14}C-citrulline.

a. ASS^- cells only
b. 97% ASS^- and 3% ASL^- cells
c. 3% ASS^- and 97% ASL^- cells
d. ASL^- cells only

8.10. EFFECT OF pH ON METABOLIC CO-OPERATION

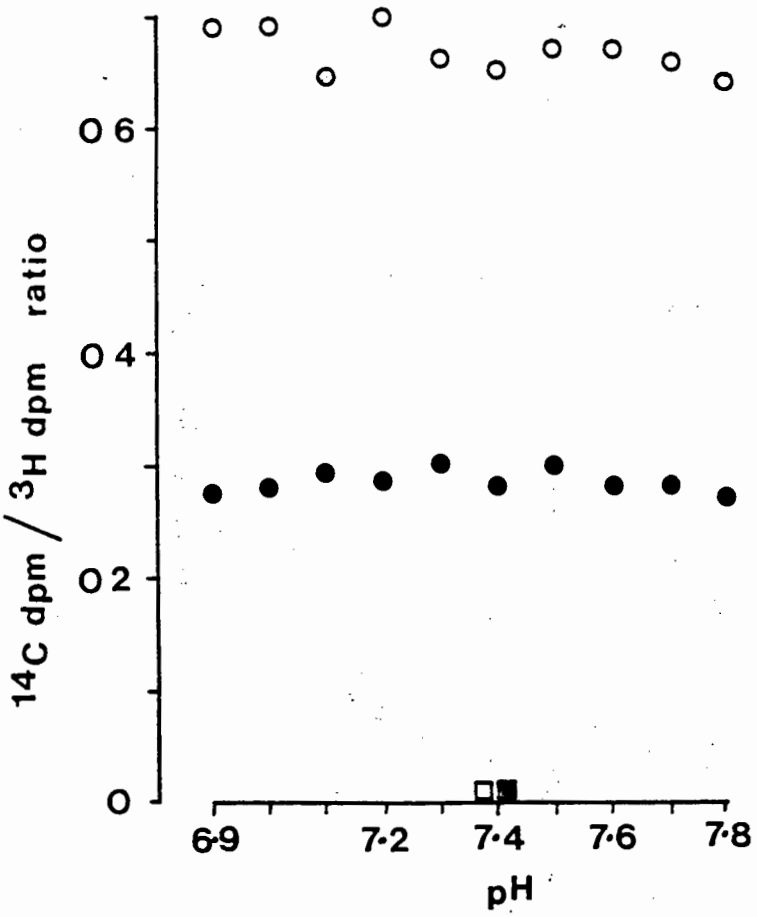
Metabolic co-operation was not sensitive to the pH of the labelling medium over the range pH 6.9 to 7.8 (Fig. 14).

8.11. EFFECT OF EXTRACELLULAR CALCIUM AND MAGNESIUM ON ESTABLISHED METABOLIC CO-OPERATION

The removal of divalent cations from the medium using EDTA is widely used to detach cells from each other and from the substratum or extracellular matrix (Adams, 1980; Muir, 1967; Berry and Friend, 1969; Urushihara et al., 1976; Lloyd et al., 1976). For this reason it was of interest to investigate the effects of removal of calcium and magnesium on intercellular junctional communication.

The effect of removing extracellular calcium and magnesium on established junctions was tested by measuring ^{14}C-citrulline incorporation relative to ^{3}H-phenylalanine in co-cultures of ASS^- and ASL^- cells 20 hours after mixing the cells (Table 10). In media lacking calcium and/or magnesium, incorporation of both isotopes by normal cells was slightly decreased. Therefore to quantitate the effect of different media on intercellular communication it is necessary to express the citrulline incorporation by the co-culture as a percentage of the value obtained in normal cells in the same medium. The co-cultures showed changes in isotope incorporation similar to the normal cells,

FIGURE 14



Effect of pH of the labelling medium on citrulline incorporation in ASS$^-$/ASL$^-$ co-cultures (●) and normal fibroblasts (O). The rate of citrulline incorporation in separate cultures of ASS$^-$ (□) and ASL$^-$ (■) cells is shown for comparison.

Labelling medium : Z (pH adjusted with HCl or NaOH)

Labelling period : 2 hours

^{14}C-citrulline : 0.2 µCi/ml

^{3}H-phenylalanine : 0.2 µCi/ml

TABLE 10

CELL LINE	MEDIUM	^{3}H DPM (PHENYLALANINE)	^{14}C DPM (CITRULLINE)	$^{14}C/^{3}H$ DPM RATIO x 100	MEAN % OF NORMAL
ASS^-	a	139584	46	0.03	0.1
		142341	46	0.03	
ASL^-	a	153523	76	0.05	0.2
		159460	66	0.04	
NORMAL CELLS	a	115812	25794	22.3	100
		124220	29876	24.1	
	b	92392	18494	20.0	100
		86277	17660	20.4	
	c	88155	21764	24.7	100
		120756	31140	25.8	
	d	78398	15214	19.4	100
		73444	14664	20.0	
ASS^-/ASL^- CO-CULTURE	a	136394	20596	15.1	64
		151440	22386	14.8	
	b	130635	18766	14.4	72
		105664	15380	14.6	
	c	156690	23442	15.0	58
		171509	24184	14.1	
	d	107629	11178	10.4	54
		81700	9010	11.0	

LEGEND TO TABLE 10

EFFECT OF REMOVING EXTRACELLULAR CALCIUM AND MAGNESIUM ON ESTABLISHED PERMEABLE JUNCTIONS

ASS^-, ASL^- and normal fibroblasts were trypsinised, suspended in fresh medium, and plated into 6 cm dishes (7×10^5 cells per dish). Co-cultures contained equal proportions of ASS^- and ASL^- cells. After 20 hours the cells were washed with calcium- and magnesium-free PBS and incubated without shaking in labelling medium containing the following additions:

a : 1 mM $CaCl_2$, 1 mM $MgCl_2$

b : 1 mM $MgCl_2$, 1 mM EGTA

c : 1 mM $CaCl_2$

d : 1 mM EDTA

Care was taken not to mechanically disrupt the cell monolayers. In the last column citrulline incorporation (relative to phenylalanine) into mutant cells and co-cultures is expressed as a percentage of the value for normal cells in the same medium.

Labelling medium : Y

Labelling period : 2 hours

^{14}C-citrulline : 0.5 µCi/ml

^{3}H-phenylalanine : 0.5 µCi/ml

indicating that intercellular communication was unaffected, or at most only slightly affected by removal of extracellular divalent cations.

Removal of calcium and/or magnesium caused morphological changes (Fig. 15). After 2 hours in medium lacking both calcium and magnesium, cells were rounded and only tenuously attached to the substratum, and appeared to be in contact with each other only by thin processes (Fig. 15d).

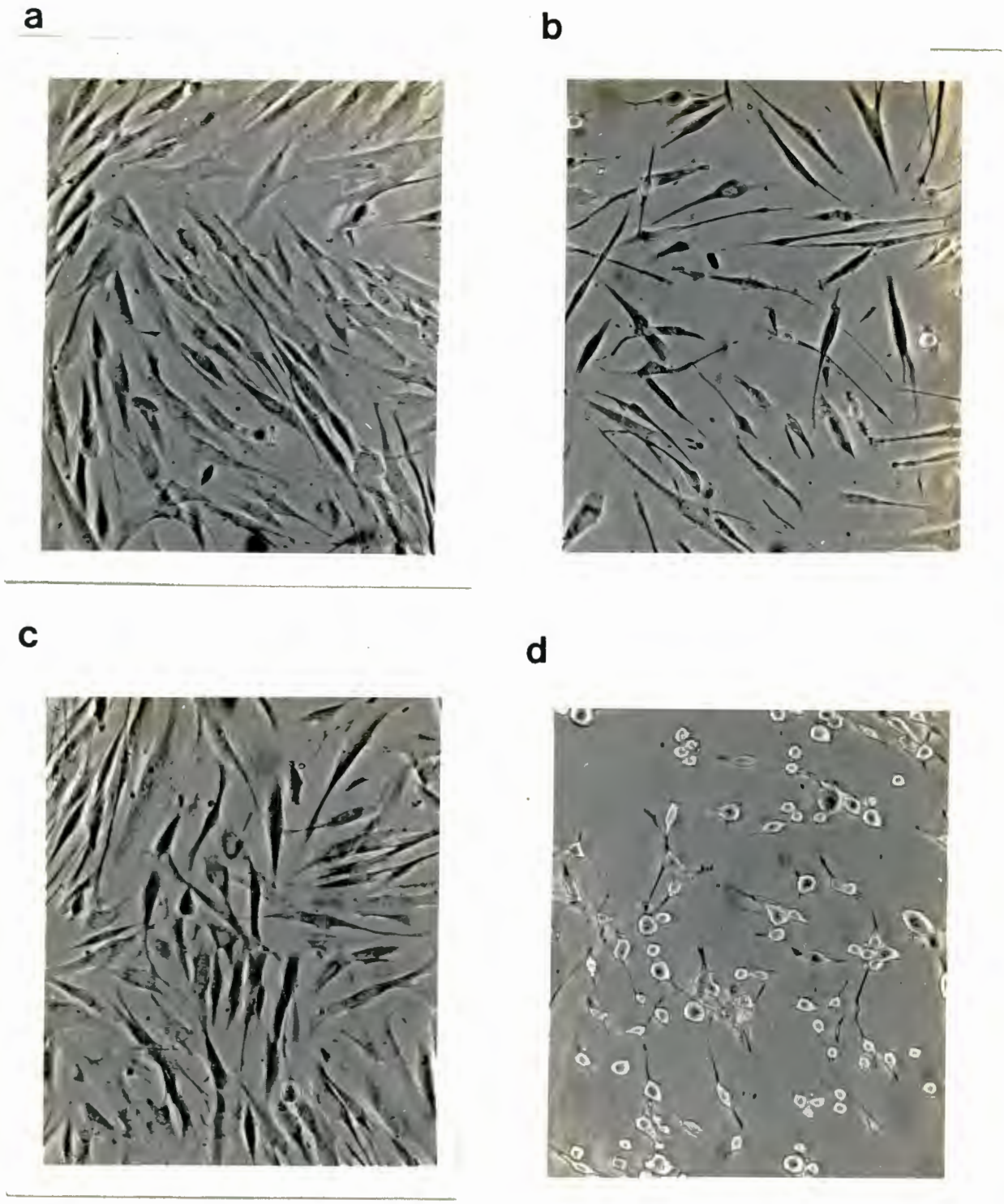
8.12. EFFECT OF CALCIUM AND MAGNESIUM ON THE FORMATION OF NEW PERMEABLE JUNCTIONS

New permeable junction formation was studied by measuring ^{14}C-citrulline incorporation in co-cultures over a 2 hour period from the time of mixing the cells (Fig. 16). Under conditions of continuous agitation, cell adhesion to the substratum did not occur to any significant extent. In the absence of calcium and magnesium the cells did not aggregate (Fig. 17) and metabolic co-operation did not occur. Metabolic co-operation increased with increasing extracellular calcium and magnesium, in association with increasing cell aggregation (Fig. 17).

The dependence of new junction formation on the concentration of calcium and magnesium was quantitatively different. At low concentrations calcium was much more effective than

magnesium in stimulating junction formation. Junction formation was half-maximal at a calcium concentration of 0.1 mM and maximal at 1 mM. The dependence of junction formation on magnesium concentration was almost linear over the range 0 to 2 mM.

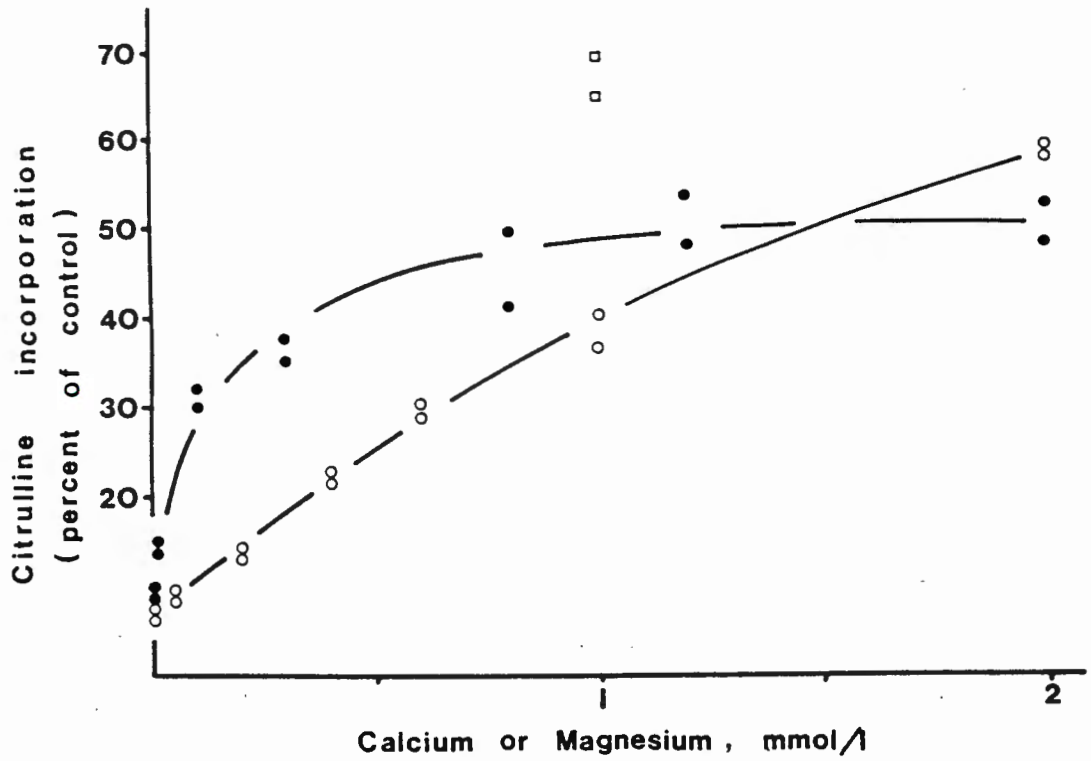
FIGURE 15



Phase-contrast micrographs of co-cultures used in the experiment shown in Table 10, taken at the end of the 2 hour labelling period. The normal cell line showed the same morphological changes.

Media a, b, c and d are described in the legend of Table 10.

FIGURE 16



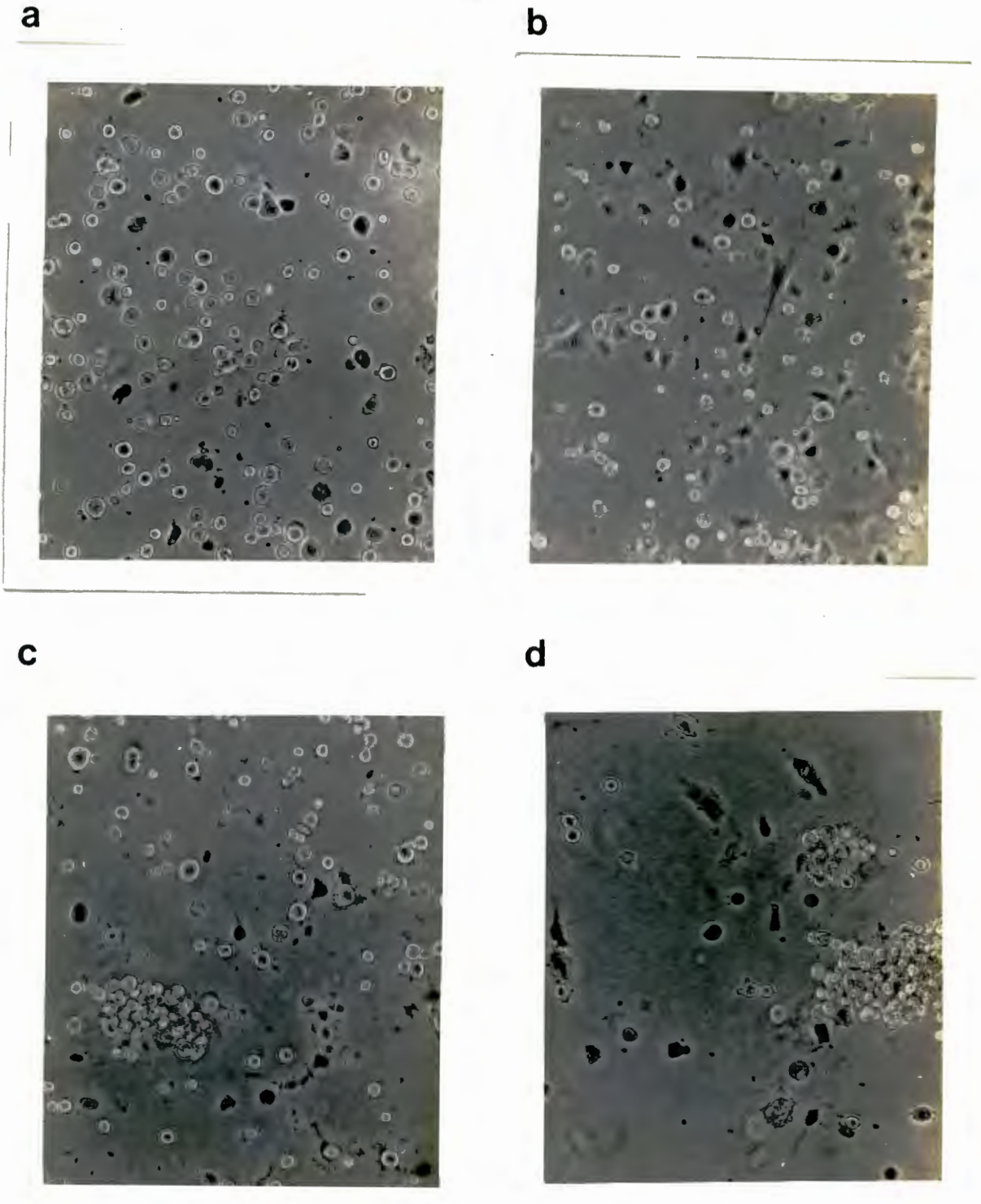
Dependence of new permeable junction formation on concentration of calcium or magnesium. ASS^-, ASL^- and normal fibroblasts were trypsinised, washed with calcium, and magnesium-free PBS, and suspended in labelling medium. The suspensions were aliquotted and varying amounts of calcium chloride and/or magnesium chloride added, after which the cells were mixed, plated into 3.5 cm dishes and incubated at 37°C with rotary shaking (120 rpm). Each dish contained 5×10^5 cells and co-cultures consisted of equal proportions of ASS^- and ASL^- cells.

- O : Varying concentration of magnesium, no added calcium, EGTA 0.2 mM.
- ● : Varying concentration of calcium, no added magnesium.
- □ : Calcium 1 mM and magnesium 1 mM.

The value plotted on the ordinate is the ^{14}C DPM / ^{3}H DPM ratio in the co-cultures expressed as a percentage of the ratio in the normal cells in the same medium.

Labelling medium : Y
Labelling period : 2 hours
^{14}C-citrulline : 0.5 µCi/ml
^{3}H-phenylalanine : 0.5 µCi/ml

FIGURE 17



Phase-contrast micrographs of co-cultures used in the same experiment shown in Fig. 16 taken at the end of the labelling period. The normal cells showed the same changes.

a. No added calcium or magnesium, 0.2 mM EDTA

b. 1 mM $MgCl_2$, 0.2 mM EGTA

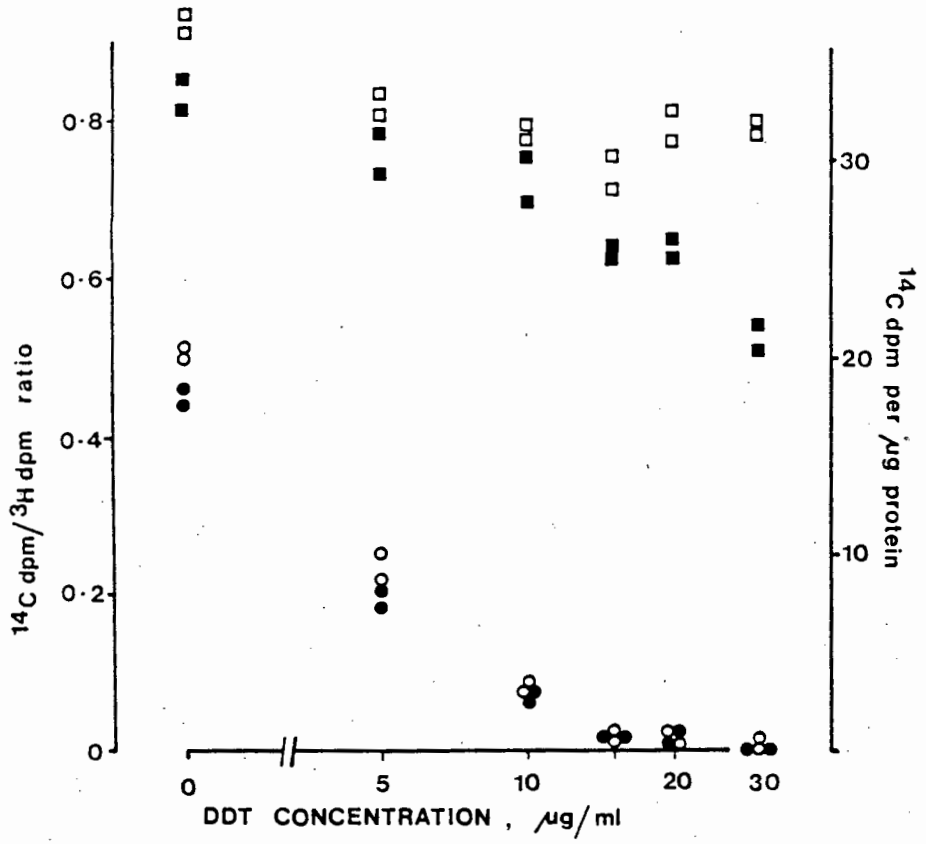
c. 1 mM $CaCl_2$

d. 1 mM $CaCl_2$, 1 mM $MgCl_2$.

8.13. INHIBITION OF METABOLIC CO-OPERATION BY DDT

In co-cultures of ASS^- and ASL^- cells, the degree of metabolic co-operation could be quantified by relating ^{14}C-citrulline incorporation either to cell protein or to ^{3}H-phenylalanine incorporation. The effect of DDT at various concentrations on metabolic co-operation expressed in both ways is shown in Fig. 18. In co-cultures, DDT inhibited citrulline incorporation in a dose-dependent fashion. Citrulline incorporation was almost completely inhibited at a DDT concentration of 20 µg/ml, with residual citrulline incorporation varying in different experiments between 1% and 10% of that without DDT. In the normal control cells having the intact citrulline metabolic pathway DDT at high concentrations caused a moderate decrease in citrulline incorporation relative to cell protein. However, phenylalanine incorporation was also decreased by a similar amount (presumably reflecting a decrease in overall protein synthesis) so that citrulline incorporation relative to phenylalanine was only slightly decreased. To correct for this effect on normal cells, the citrulline/phenylalanine incorporation ratio in co-cultures was expressed as a percentage of the ratio in control cells. This value was taken as a measure of metabolic co-operation. Fig. 19 illustrates the use of this approach and contrasts the effect on metabolic co-operation of DDT with that of 2,4-dinitrophenol, an inhibitor of cellular energy metabolism. 2,4-Dinitrophenol decreased citrulline incorporation in co-cultures and normal

FIGURE 18



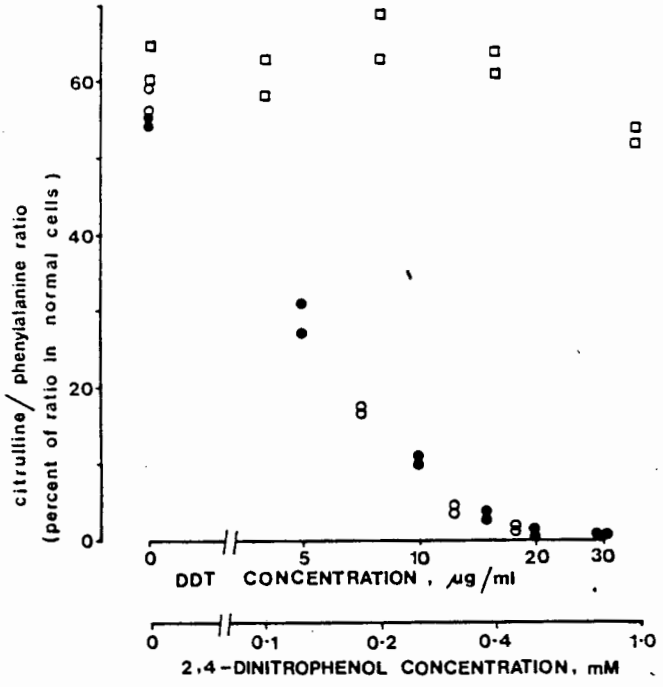
Citrulline incorporation in co-cultures (O,●) and normal cells (□,■) at different concentrations of DDT.

O , □ : ^{14}C DPM/^{3}H DPM RATIO.

● , ■ : ^{14}C DPM per µg cell protein.

DDT was added to the cells immediately after plating, 20 hours before the start of the labelling period. Labelling was for 2 hours in medium A containing ^{14}C-citrulline (0.5 µCi/ml) and ^{3}H-phenylalanine (1 µCi/ml) and DDT at the concentrations shown. The values from duplicate dishes are shown.

FIGURE 19



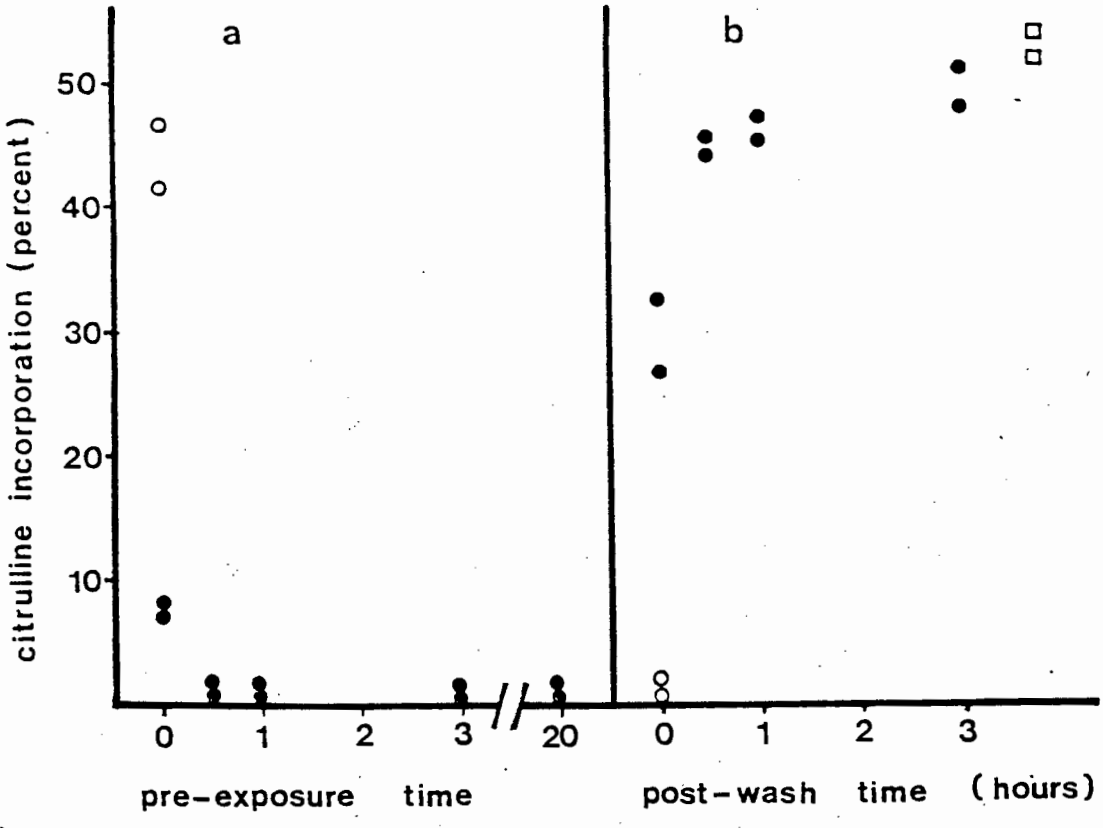
Comparison of the effects of DDT and 2,4-dinitrophenol on intercellular communication. The citrulline/phenylalanine incorporation ratio in co-cultures as a percentage of the ratio in normal cells is plotted as a function of the concentration of DDT (○ , ● representing data from two experiments) and 2,4-dinitrophenol (□). Labelling conditions were as described for Fig. 18. The values for duplicate dishes are shown.

cells to the same degree at concentrations up to 1 mM, which resulted in a 75% decrease in absolute citrulline incorporation; the citrulline/phenylalanine ratio in co-cultures therefore remained a constant percentage of that in the normal cells.

DDT was effective in inhibiting metabolic co-operation whether added at the time of plating the cells or several hours later (Fig. 20a), indicating that it acts on established junctions and not only on new junction formation. The onset of the DDT effect was rapid. The duration of the labelling period limits the determination of the rapidity with which inhibition becomes established. However, when exposure to DDT at a concentration of 20 μg/ml was commenced at the start of the labelling period, inhibition of metabolic co-operation was almost as great as when the co-cultures were exposed to DDT before labelling. This indicates that the time taken to establish inhibition of metabolic co-operation by DDT is substantially less than one hour. There was no tendency for the inhibition to diminish with time since co-cultures exposed to DDT at 15 μg/ml for 3 days still showed an inhibition of metabolic co-operation of greater than 96% (Table 11).

The effect of DDT was reversible. Fig. 20b shows the effect of washing the DDT-containing medium off the cells before labelling. The greater part of the inhibition caused by DDT was reversed when the DDT-containing medium was decanted and the cells washed immediately before labelling. Metabolic

FIGURE 20



Time-course of the onset and reversal of inhibition of metabolic co-operation by DDT. The ordinate shows the citrulline/phenylalanine ratio in co-cultures as a percentage of the ratio in normal cells.

(a) Cells were plated 26 hours before the start of labelling. To the dishes shown as closed circles (●) DDT (20 µg/ml) was added at the indicated times before the start of labelling and was also present during the labelling period. The dishes shown as open circles (○) were not exposed to DDT. Labelling was for 1 hour in Medium Z containing ^{14}C-citrulline (0.3 µCi/ml) and ^{3}H-phenylalanine (0.3 µCi/ml).

(b) Cells were plated 22 hours before the start of labelling and DDT (20 µg/ml) was added to the dishes shown as circles immediately. At the indicated times before the start of labelling, the medium was poured off, the cells were washed once with Dulbecco's phosphate-buffered saline and fresh medium without DDT was added. The dishes shown as open circles (○) were not washed, and were labelled in the presence of DDT. The dishes shown as squares (□) were not exposed to DDT. Labelling was for 1.5 hours in medium as described in (a) above.

TABLE 11

CULTURE	DDT	^{3}H dpm	^{14}C dpm	$\frac{^{14}C \text{ dpm}}{^{3}H \text{ dpm}} \times 100$
CO-CULTURE	-	75433	9868	13.1
		65250	8077	12.4
	+	89658	166	0.19
		87193	166	0.19
NORMAL CELLS	-	48431	11273	23.3
		42145	9579	22.8
	+	62224	15482	24.9
		56566	12755	22.6

Effect of prolonged exposure to DDT on metabolic co-operation. Co-cultures of equal proportions of ASS^- and ASL^- cells, and cultures of normal cells were plated, and DDT was added, 3 days before labelling.

Labelling medium : Z

Labelling period : 1.5 hours

^{14}C-citrulline : 0.25 µCi/ml

^{3}H-phenylalanine : 0.25 µCi/ml

co-operation approached normal levels when the DDT was washed off 30 min before labelling.

Increased cytoplasmic free calcium concentration is known to inhibit junctional communication in some cell types (Rose et al., 1977; Loewenstein, 1981). It has been proposed that DDT inhibits intercellular communication by inhibiting the Ca,Mg-ATPase of the plasma membrane and thereby increasing intracellular free calcium (Madhukar et al., 1983). This was tested by measuring metabolic co-operation in the absence of extracellular calcium, and it was found that inhibition of metabolic co-operation by DDT was not dependent on the presence of free calcium in the medium (Table 12). Even assuming that the washing were to remove only 90% of the original calcium, the presence of 2 mM EGTA would decrease the free calcium in the medium to less than 10^{-9} M (Vianna, 1975) which is less than the intracellular calcium concentration. Under these conditions inhibition of the plasma membrane Ca,Mg-ATPase would not result in an increase in intracellular calcium, yet DDT still inhibited metabolic co-operation. This indicates that the effect of DDT on junctional communication is independent of its effect on the Ca,Mg-ATPase.

DDT at concentrations up to 20 µg/ml did not demonstrably affect cell multiplication over a period of 6 days (Fig. 21). At 30 µg/ml cell multiplication was inhibited. At 20 µg/ml the appearance of the cells by phase-contrast microscopy was unchanged, except for the presence of dark granules in the

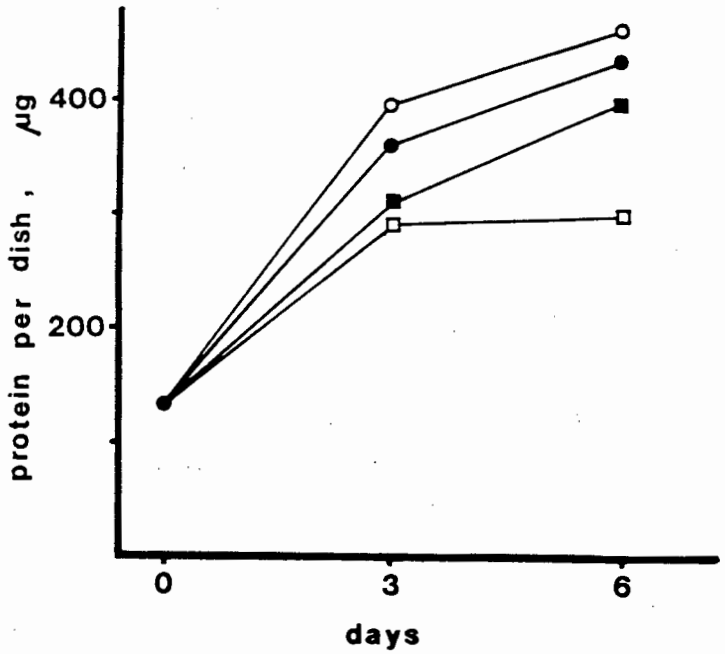
TABLE 12

			^{3}H DPM x 10^{-3}	^{14}C DPM x 10^{-3}	^{14}C / ^{3}H	$^{14}C/^{3}H$ in co-culture as % of normal cells
Normal Cells	2 mM Ca	Ethanol	237	99.1	0.418	
			265	105	0.398	
		DDT	172	65.4	0.380	
			192	73.2	0.381	
	2 mM EGTA	Ethanol	188	66.7	0.355	
			167	60.1	0.360	
		DDT	122	45.9	0.378	
			133	47.2	0.355	
ASS^{-}/ASL^{-} Co-culture	2 mM Ca	Ethanol	296	56.4	0.191	46.8
			316	53.8	0.171	41.8
		DDT	237	3.8	0.016	4.2
			234	2.7	0.011	3.0
	2 mM EGTA	Ethanol	222	24.0	0.108	30.2
			238	24.6	0.103	28.8
		DDT	176	2.2	0.013	5.5
			177	2.0	0.011	4.2

LEGEND TO TABLE 12

Effect of DDT on metabolic co-operation in the presence and absence of extracellular free calcium. Normal cells and co-cultures were innoculated at a density of 10^6 cells per 60 mm dish and 20 hours later washed once with medium Y without calcium. They were then incubated for 15 min in 3 ml medium Y containing 1 mM $MgCl_2$ with either DDT in ethanol or ethanol alone and either 2 mM $CaCl_2$ or 2 mM EGTA, after which the media were replaced with 1.5 ml of the same media containing ^{14}C-citrulline (0.4 µCi/ml) and ^{3}H-phenylalanine (0.4 µCi/ml). Incubation was continued for 1.5 hours after which radioactivity in TCA-insoluble material was determined.

FIGURE 21



Growth of co-cultures in media containing various concentrations of DDT. DDT in ethanol or ethanol alone (1% v/v) was added to Ham's F10 medium containing 10% foetal calf serum. The medium was renewed after 3 days.

● : Ethanol only

O : DDT 10 µg/ml

■ : DDT 20 µg/ml

□ : DDT 30 µg/ml

The mean value of duplicate dishes is shown.

cytoplasm after several hours of exposure to DDT. There was no rounding-up of the cells or detachment from the substratum.

The inhibition of metabolic co-operation by DDT was not sensitive to the ratio of ASS^- to ASL^- cells in the co-culture: similar inhibition was observed in co-cultures containing 20%, 50% and 80% of cells of one type (Table 13).

TABLE 13

PERCENTAGE OF CELL TYPE					
ASS^-	ASL^-	DDT	^3H dpm (Phenyl-alanine)	^{14}C dpm (Citrulline)	$\frac{^{14}C \text{ dpm}}{^3H \text{ dpm}} \times 100$
20	80	+	99607	191	0.19
			95707	111	0.12
		-	103700	14517	14.0
			114884	14162	12.3
50	50	+	105999	264	0.25
			106787	171	0.16
		-	110456	17275	15.6
			108424	17464	16.1
80	20	+	110763	166	0.15
			103554	142	0.14
		-	110333	11906	10.8
			109419	11342	10.4
100	0	-	107727	31	0.03
0	100	-	135740	20	0.01

Inhibition of metabolic co-operation by DDT (20 µg/ml) in co-cultures consisting of different proportions of ASS^- and ASL^- cells.

Labelling medium : Z
Labelling period : 1.5 hours
^{14}C-citrulline : 0.3 µCi/ml
^3H-phenylalanine : 0.3 µCi/ml

8.14. THE USE OF CHINESE HAMSTER V79 CELLS IN CO-CULTURE WITH HUMAN ASL$^-$ FIBROBLASTS TO MEASURE JUNCTIONAL COMMUNICATION

Since Chinese hamster V79 cells are known to be deficient in ASS (Gonzalez-Noreiga et al., 1980) it was of interest to determine whether these cells would show metabolic co-operation with human ASL$^-$ fibroblasts.

Table 14 shows incorporation of ^{14}C-citrulline relative to ^{3}H-phenylalanine into TCA-insoluble material by the cell lines separately, and by the two types of co-culture. As previously shown (Fig. 7) co-cultures of ASS$^-$ and ASL$^-$ fibroblasts (referred to as ASS$^-$/ASL$^-$ co-cultures) incorporate citrulline at rates at least 100 fold greater than each cell line separately. Co-cultures of V79 and ASL$^-$ cells (V79/ASL$^-$ co-cultures) incorporate citrulline at rates 20 to 50 fold higher than V79 and ASL$^-$ cells separately (Table 14). Thus metabolic co-operation occurs between these latter cell types, in a similar fashion to that between ASS$^-$ and ASL$^-$ human fibroblasts, but to a lesser degree, as judged by the rate of citrulline incorporation.

TABLE 14

Cell line	^{3}H-phenylalanine dpm	^{14}C-citrulline dpm	^{14}C/^{3}H ratio x 1000
ASS$^-$	193731	44	0.23
fibroblasts	233174	44	0.18
ASL$^-$	425722	103	0.24
fibroblasts	399441	113	0.28
V79	502031	100	0.20
	498570	93	0.19
ASS$^-$/ASL$^-$	332389	15144	45.6
co-culture	322677	14931	46.3
V79/ASL$^-$	827644	4162	5.03
co-culture	881666	3717	4.22

Incorporation of ^{14}C-citrulline and ^{3}H-phenylalanine into trichloroacetic acid-insoluble material by cell lines separately and in co-cultures.

Labelling medium : Z
Labelling period : 2 hours
^{14}C-citrulline : 0.4 µCi/ml
^{3}H-phenylalanine : 0.2 µCi/ml

8.15. EFFECT OF TPA ON METABOLIC CO-OPERATION

The effect of short-term exposure to TPA on citrulline incorporation in the two types of co-culture is shown in Figure 22. In V79/ASL$^-$ co-cultures TPA inhibited citrulline incorporation by 50% at a concentration of 0.25 - 0.4 ng/ml and by 95-98% at 10 ng/ml. Phenylalanine incorporation, reflecting overall protein synthesis, was unaffected by TPA. In normal fibroblasts, both absolute phenylalanine incorporation and the citrulline/phenylalanine ratio were unaffected by TPA. This indicates that TPA is acting at the level of intercellular junctional communication and not at some other step in the process of citrulline incorporation into protein. A more rigorous explanation of this assertion is given in Section 9.6.

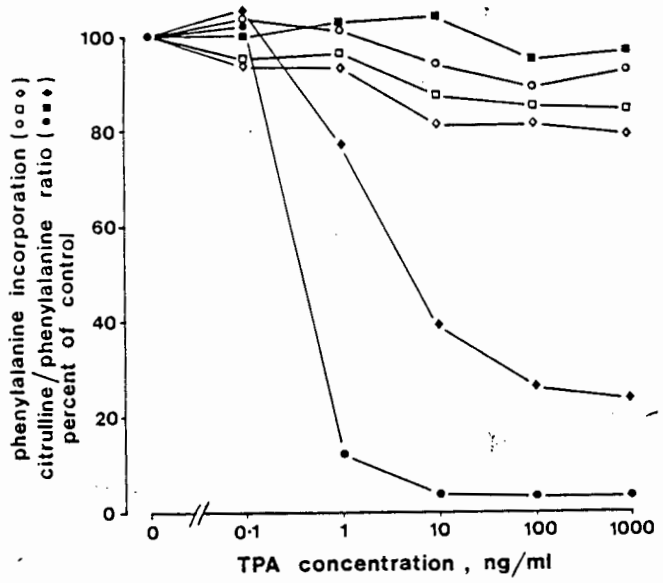
In ASS$^-$/ASL$^-$ co-cultures, metabolic co-operation was less sensitive to inhibition by TPA. Citrulline incorporation was inhibited by 50% at a TPA concentration of 5 ng/ml, and inhibition was never greater than 80%, even at 1000 ng/ml (Figure 22).

In the experiments described above the cells were exposed to TPA for 30 minutes before labelling: similar experiments in which cells were exposed to TPA for 19 hours before labelling showed that the degree of inhibition of junctional communication was not increased by longer exposure to TPA. In fact, at TPA concentrations of 100 ng/ml or greater, the inhibition was reversed after long exposure (Figure 23). This

refractoriness was observed in both types of co-culture, and took at least 4 hours to develop. Co-cultures which had been exposed to a high concentration of TPA for prolonged periods and then washed showed an intermediate degree of metabolic co-operation, varying between 16% and 40% of control in different experiments (Table 15). These cells were no longer responsive to further addition of TPA at the lower doses which normally inhibited metabolic co-operation. This state persisted for at least 4 hours after washing (Table 15). Co-cultures in this refractory state were nevertheless still sensitive to communication inhibition by RA or DDT (Table 15).

When co-cultures were treated with TPA (10 ng/ml) and then washed, a slow progressive reversal of inhibition of metabolic co-operation was observed: however metabolic co-operation was still inhibited by 60% at 19 hours after washing (Figure 24).

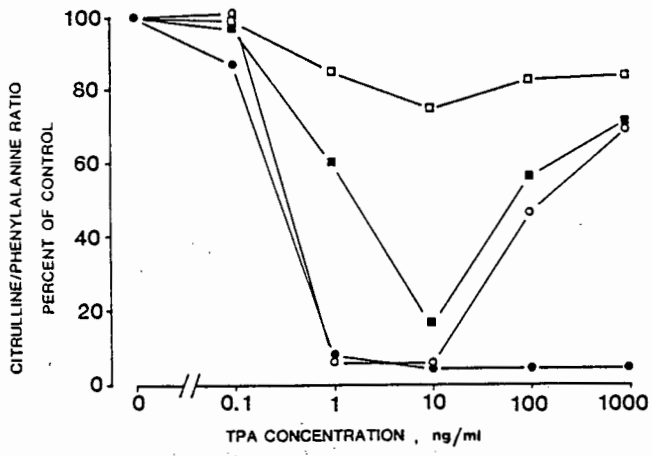
FIGURE 22



Effect of short exposure to TPA on metabolic co-operation. ASS^-/ASL^- co-cultures (◆,◇), V79/ASL^- co-cultures (●,○) and normal human fibroblasts (■,□) were treated with TPA in ethanol or ethanol alone for 30 min before incubating for 2 hours in labelling medium containing TPA or ethanol, ^{14}C-citrulline and ^{3}H-phenylalanine. The ^{14}C-dpm/^{3}H-dpm ratios (◆,●,■) are expressed as a percentage of the ratio in the appropriate ethanol control. The absolute phenylalanine incorporation (◇,○,□), expressed as a percentage of control, serves as a measure of overall protein synthesis. Each point is the mean of duplicate determinations, which in all cases differed by less than 12%.

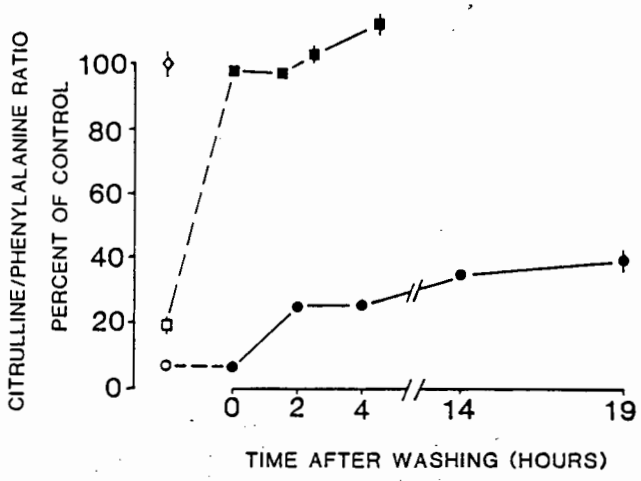
For labelling conditions see Section 7.1.

FIGURE 23



Effect of long exposure to TPA on metabolic co-operation. Symbols are as follows: □ normal cells, 19 hour exposure; ● V79/ASL$^-$ co-cultures, 4 hour exposure; ○ V79/ASL$^-$ co-cultures, 19 hour exposure; ■ ASS$^-$/ASL$^-$ co-cultures, 19 hour exposure. The data points represent the citrulline/phenylalanine ratios expressed as percent of the ratios without TPA. Each point is the mean of duplicates which differed in all cases by less than 15%. Labelling conditions were as for Figure 22.

FIGURE 24



Reversibility of inhibition of metabolic co-operation by TPA and RA. TPA (10 ng/ml) was added to co-cultures (○,●) 23 hours before labelling. RA (10^{-5} M) was added to co-cultures (□,■) 5.5 hours before labelling. At the indicated times before labelling the cells (●,■) were washed 3 times and incubation was continued in medium without TPA or RA; these co-cultures were labelled in the absence of TPA or RA. Other co-cultures (○,□) were not washed and were labelled in the presence of TPA (10 ng/ml) and RA (10^{-5} M) respectively. Citrulline/phenylalanine ratios in co-cultures are expressed as a percentage of the ratio in control co-cultures (◇) which were not exposed to TPA or RA at any stage. Labelling conditions were as in Figure 22.

TABLE 15

	Pre-treatment	Labelling medium	Citrulline/phenylalanine ratio (percent + difference)
Experiment 1	-	-	100 ± 3.8
	-	TPA 10 ng/ml	2.4 ± 0.3
	500 ng/ml TPA for 22 h	-	37 ± 0.5
	500 ng/ml TPA for 22 h	TPA 10 ng/ml	40 ± 5.7
	500 ng/ml TPA for 18 h, wash, recovery for 4 h	TPA 10 ng/ml	33 ± 2.0
Experiment 2	-	-	100 ± 9.7
	-	TPA 25 ng/ml	2.6 ± 1.1
	-	RA 10 µM	1.7 ± 0.2
	-	DDT 20 µM	67 ± 2.5
	-	DDT 50 µM	7.6 ± 0.8
	1000 ng/ml TPA for 20 h	-	16.1 ± 0.6
	1000 ng/ml TPA for 20 h	TPA 25 ng/ml	17.1 ± 5.3
	1000 ng/ml TPA for 20 h	RA 10 µM	1.1 ± 0.2
	1000 ng/ml TPA for 20 h	DDT 20 µM	6.0 ± 1.3
	1000 ng/ml TPA for 20 h	DDT 50 µM	1.3 ± 0.3

LEGEND TO TABLE 15

Desensitisation of co-cultures after prolonged exposure to high concentrations of TPA. Co-cultures were plated 26 hours before labelling. During this period some co-cultures were exposed to high concentrations of TPA as indicated. All co-cultures were then washed with medium without TPA, and labelled for 2 hours with or without TPA, RA or DDT, as indicated. The figures are the citrulline/phenylalanine ratios (mean $\pm$ difference, of duplicate dishes) expressed as a percentage of the ratio in control co-cultures not exposed to any of the compounds. For labelling conditions see Section 7.1.

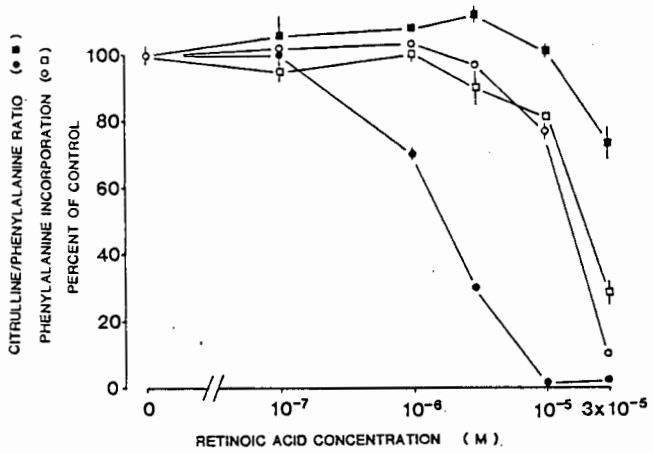
8.16. EFFECT OF RETINOIDS ON METABOLIC CO-OPERATION

Exposure to retinoic acid (RA) for 30 min prior to labelling inhibited metabolic co-operation in both types of co-culture to a similar degree (Figures 25 and 26). Fifty percent inhibition was reached at a RA concentration of 0.5 to 1.7 μM and 98% inhibition at 10 μM. At concentrations greater than 10 μM, RA inhibited phenylalanine incorporation in both co-cultures and normal cells, indicating a toxic effect on the cells resulting in decreased protein synthesis. A moderate decrease in citrulline/phenylalanine ratio in normal cells occurred at toxic concentrations of RA (Figure 25) indicating an inhibitory effect on some stage in the citrulline metabolic pathway. However this decrease in normal cells was never observed at non-toxic concentrations. Longer exposure (19 hours) to RA resulted in less inhibition of metabolic co-operation at any given RA concentration: however, virtually complete inhibition was still observed at high RA concentrations, and refractoriness of the type seen with TPA did not occur (Figure 26).

The effects of RA, retinal, retinol and retinyl acetate on ASS^-/ASL^- co-cultures are summarised for comparison in Table 16. Retinal inhibited metabolic co-operation with equal potency to RA. Retinol was 10 fold less potent and retinyl acetate was 30 fold less potent, causing 50% inhibition only at toxic concentrations.

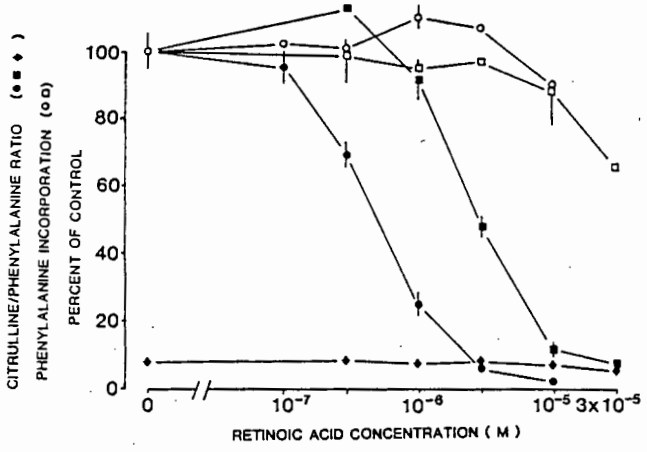
Rapid and complete reversal of inhibition occurred when co-cultures which had been treated with RA (10^{-5} M) were washed (Figure 24). No residual inhibition could be detected even when cells were labelled immediately after washing.

FIGURE 25



Effect of retinoic acid on normal fibroblasts (■, □) and ASS^-/ASL^- co-cultures (●, ○). Cells were exposed to RA for 30 minutes before labelling and during the 2-hour labelling period. Citrulline/phenylalanine ratios (●, ■) and absolute phenylalanine incorporation (○, □) are expressed as percent of the DMSO-treated controls. For labelling conditions see Section 7.1.

FIGURE 26



Effect of short and long exposure to retinoic acid on V79/ASL$^-$ co-cultures. Cultures (●, ○) were exposed to RA for 30 minutes before labelling and during the 2-hour labelling period. Cultures (■, □) were exposed to RA for 19 hours before labelling and during labelling. Cultures (◇) were exposed to RA for 19 hours before labelling, and during the labelling period, and TPA (20 ng/ml) was added immediately before labelling. Citrulline/phenylalanine incorporation ratios (●, ■, ◆) and absolute phenylalanine incorporation (○, □) are expressed as percent of controls without RA or TPA. For labelling conditions see Section 7.1.

TABLE 16

	50% inhibitory concentration (μM)	98% inhibitory concentration (μM)	Toxic concentration (μM)
Retinoic acid	1.7	10	16
Retinal	1.9	10	7.1
Retinol	20	100	63
Retinyl acetate	63	-	56

Effects of retinoids on metabolic co-operation in ASS^-/ASL^- co-cultures. The 50% and 98% inhibitory concentrations are the concentrations at which the citrulline/phenylalanine incorporation ratio in co-cultures was decreased by 50% and 98% of the ratio in the DMSO-treated control co-cultures. The toxic concentration is the concentration at which phenylalanine incorporation in co-cultures was decreased by 50%. These values were determined from dose-response curves of the type shown in Fig. 25.

8.17. EFFECTS OF SIMULTANEOUS EXPOSURE TO TPA AND RA OR FLUOCINOLONE ACETONIDE ON METABOLIC CO-OPERATION

Since metabolic co-operation in V79/ASL$^-$ co-cultures was more sensitive to inhibition by TPA than in ASS$^-$/ASL$^-$ co-cultures, the former system was used to investigate whether any interaction could be detected between retinoic acid and TPA. Figure 27 shows the effects on metabolic co-operation of simultaneous exposure to varying concentrations of TPA and retinoic acid. At concentrations at which RA did not itself inhibit metabolic co-operation, RA had no effect on the inhibition of metabolic co-operation by TPA. As the retinoic acid concentration was increased, the inhibitory effects of the two compounds became superimposed in a simple additive fashion. There was no evidence of antagonism or of synergism between these two compounds with respect to inhibition of metabolic co-operation. A similar lack of antagonism was seen when co-cultures were treated with RA for 19 hours before adding TPA (Figure 26).

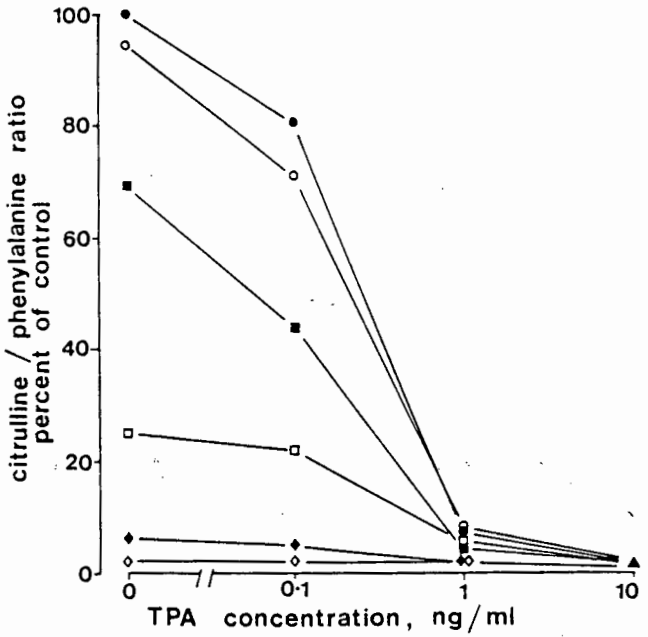
Fluocinolone acetonide at high concentration (125 μM) did not antagonise the inhibitory effect of TPA on metabolic co-operation (Table 17).

TABLE 17

	CITRULLINE/PHENYLALANINE RATIO (percent of control)
CONTROL	100 ± 2.2
TPA	2.5 ± 0.8
FA (30 min)	71 ± 9.0
FA (30 min) + TPA	2.6 ± 1.1
FA (18 hours)	102 ± 15.8
FA (18 hours) + TPA	2.8 ± 1.1

Effects of TPA (25 ng/ml) and fluocinolone acetonide (FA) (125 μM) on metabolic co-operation in V79/ASL$^-$ co-cultures. TPA was added 30 minutes before the start of the labelling period and was also present in the labelling medium where indicated. FA was added either 30 minutes or 18 hours before labelling and was also present during the labelling period where indicated. Results are mean ± difference of duplicate dishes, as percent of control.

FIGURE 27



Effect of TPA and RA simultaneously on metabolic cooperation. TPA and either RA (○, 10^{-7} M; ■, 3 x 10^{-7} M; □, 10^{-6} M; ◆, 3 x 10^{-6} M; ◇, 10^{-5} M) or 0.5% DMSO (●) were added to the cultures 30 minutes before labelling and were also present during the 2-hour labelling period. The curves shown are the citrulline/phenylalanine ratios as a percentage of the ratio without TPA and RA. Each point is the mean of duplicates which differed by less than 15% in all cases. For labelling conditions see Section 7.1.

9. DISCUSSION

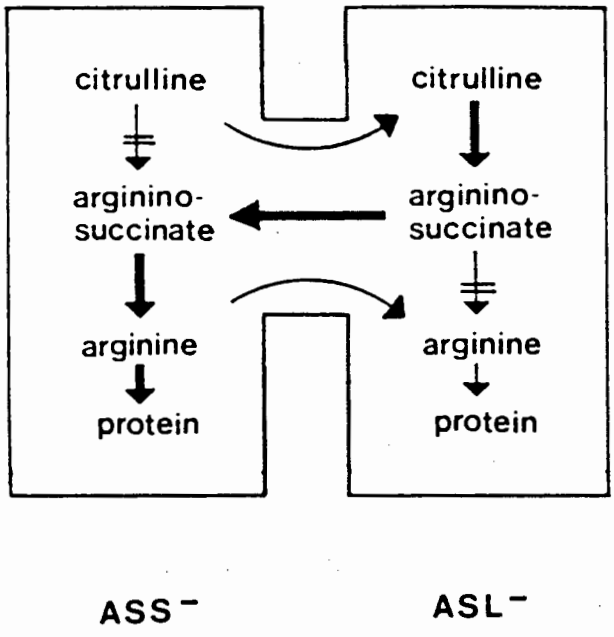
9.1. COMPLEMENTATION BETWEEN ASS$^-$ AND ASL$^-$ CELLS

It has been previously noted (Gonzalez-Noreiga et al., 1980; Cathelineau, 1981) that ASS$^-$ and ASL$^-$ cells show complementation with respect to citrulline incorporation. Gonzalez-Noreiga et al. (1980) used an autoradiographic method to show citrulline incorporation following polyethyleneglycol-induced cell fusion of Chinese Hamster V79 cells and ASL$^-$ human fibroblasts. They observed labelling only in heterokaryocytes, and apparently did not detect complementation in unfused co-cultures. Cathelineau et al. (1981) described complementation in co-cultures of human ASS$^-$ and ASL$^-$ fibroblasts without the necessity for cell fusion. They speculated that this complementation occurred by diffusion of argininosuccinate between the two cell types, but did not elaborate further on the mechanism of this transfer.

9.2. THE MECHANISM OF COMPLEMENTATION: INTERCELLULAR JUNCTIONAL COMMUNICATION

A likely mechanism for the complementation observed between these human mutant fibroblast cell lines can be inferred from the results presented in Sections 8.5 to 8.9. The dependence of complementation on cell density (Fig. 12) shows that it requires intercellular contact. The absence of demonstrable transfer of labelled intermediates through the medium (Tables 6 and 7) implies that if transfer of a metabolic intermediate between the two cell types occurs, it is through intercellular junctions which are not leaky to the outside. The obvious candidate for such an intermediate is argininosuccinate, and large amounts of added unlabelled argininosuccinate caused only a small decrease in ^{14}C-citrulline incorporation (Table 8). This indicates that the ^{14}C-argininosuccinate passes directly between cells and not via the medium in any significant quantity, where it would be diluted by the unlabelled argininosuccinate at least 100-fold. Unlabelled argininosuccinate caused a similar small decrease in ^{14}C-citrulline incorporation in a normal cell line (Table 8) showing that the decrease seen in the co-culture is probably due to intracellular dilution of ^{14}C-argininosucciante. The mechanism shown in Fig. 28 is therefore proposed: in the ASL$^-$ cells ^{14}C-citrulline is converted to ^{14}C-argininosuccinate which passes through intercellular junctions to the ASS$^-$ cells where it is converted into ^{14}C-arginine. The ^{14}C-arginine is incorporated into protein in the ASS$^-$ cells, and some of the ^{14}C-arginine

FIGURE 28



Proposed mechanism of metabolic co-operation.

passes back through the junctions to the ASL^- cells to be incorporated into protein. The evidence for the latter is the lighter labelling of neighbouring cells relative to the central heavily labelled cells seen in the autoradiographs (Fig. 13C).

It seems likely that citrulline is transported into the ASS^- cells normally and that the passage of this citrulline via the junctions to the ASL^- cells further augments the degree of metabolic co-operation, although there is no direct evidence in support of this point.

The finding that ASL-deficient lymphoblasts did not show metabolic co-operation with ASS^- fibroblasts supports the conclusion that metabolic co-operation does not occur by simple diffusion of a metabolite into the medium, and indicates that EBV-transformed lymphoblasts do not form intercellular junctions with fibroblasts. In this respect EBV-transformed lymphoblasts do not differ from unstimulated or PHA-stimulated lymphocytes, which have been reported not to show metabolic co-operation (Cox et al., 1974).

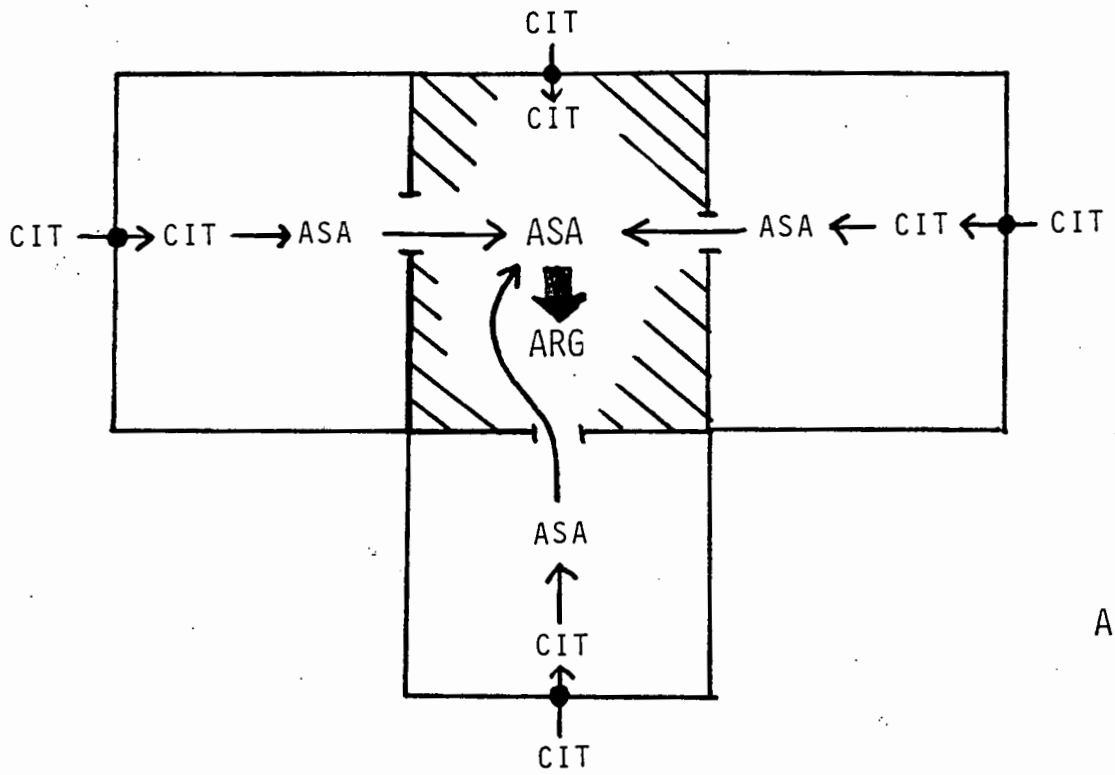
The mechanism of complementation as proposed here is consistent with the known properties of gap-junctional intercellular communication (discussed in Sections 6 to 6.3): the molecular weights of argininosuccinate, citrulline and arginine are 290, 175 and 174 respectively, so that these molecules are certainly small enough to pass through gap junctions.

An alternative explanation which must be considered is that complementation results from the exchange of enzymes rather than metabolites. Enzyme exchange between cells in culture is well described for the lysosomal enzymes involved in mucopolysaccharide degradation (Neufeld 1974). Uptake of cytosolic and mitochondrial enzymes by human fibroblast cultures has also been reported (Ashkenazi and Gartler, 1971). However, this type of transfer occurs by the release of the enzyme into the medium and subsequent uptake by the deficient cell type. Cell contact is not necessary: the defect in one cell type can be corrected by treating the cells with medium in which the other cell type has been cultured, or with soluble cell extracts. The results of Sections 8.6, 8.8 and 8.12 show that exchange via the medium does not occur in the system described here. The enzymes ASS and ASL have subunit molecular weights of 45,000 and 49,000 respectively (Rochovansky et al., 1977; Kimball and Jacoby, 1980; Ratner, 1973; O'Brien and Barr, 1981); these enzymes are thus far too large to be transmitted via gap junctions. Therefore if complementation occurs by the exchange of ASS and ASL enzymes, this would require intercellular junctions which were permeable to macromolecules. Since there is no evidence for the existence of such junctions, this alternative mechanism is highly unlikely.

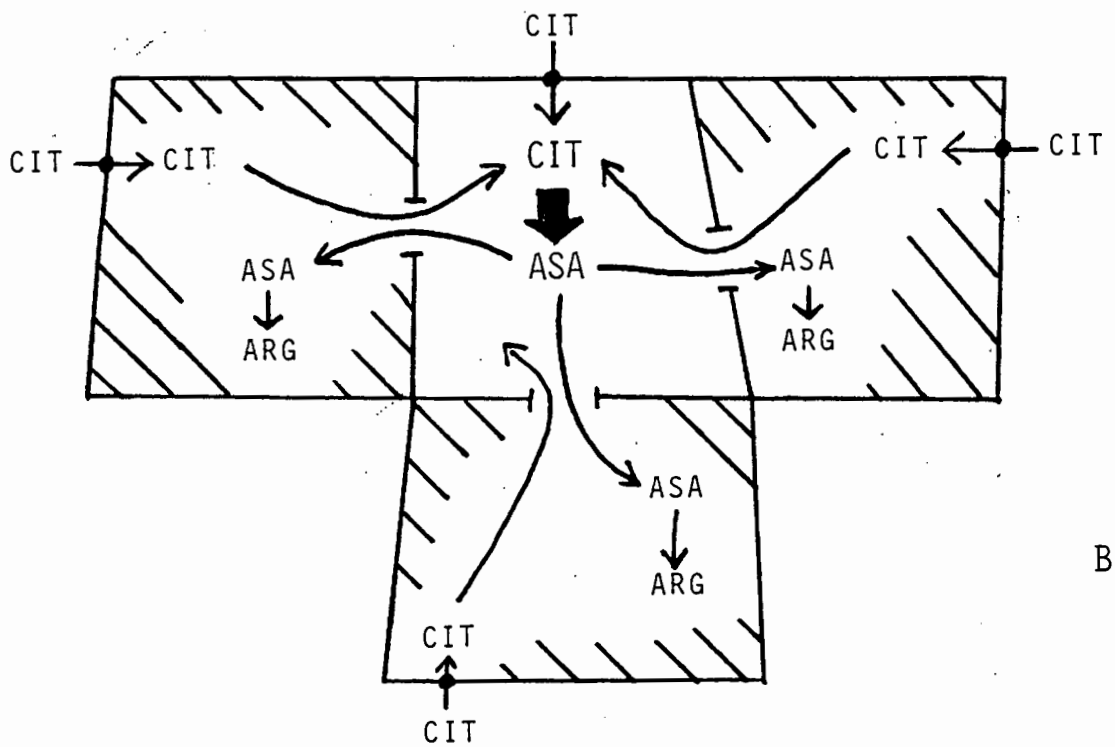
9.3. QUANTITATIVE FEATURES OF METABOLIC CO-OPERATION BETWEEN ASS$^-$ AND ASL$^-$ CELLS

An interesting feature of this metabolic co-operation system is the fact that the rate of citrulline incorporation relative to leucine can be as high in co-cultures as in normal fibroblasts, even when only 6% of the cells in the co-culture contain ASL. This results in the skewed shape of the curve shown in Fig. 8A. This skewness implies that in co-cultures containing only a small percentage of ASS$^-$ cells, these latter cells have the capacity to convert efficiently to arginine all the argininosuccinate supplied by the excess of ASL$^-$ cells. This results in a flux of argininosuccinate to arginine through each ASS$^-$ cell which is up to 20-fold greater than in normal cells under the same labelling conditions (Fig. 8B). Under these labelling conditions, with no additional unlabelled citrulline (total citrulline concentration : 0.01 mmol/ℓ), the citrulline pathway is operating well below its maximal capacity (Figs. 3 and 10). Half-maximal citrulline incorporation is achieved at a citrulline concentration of 0.2 mmol/ℓ. When a small number of ASS$^-$ cells are co-cultured with a large number of ASL$^-$ cells, the flux per ASS$^-$ cell can increase up to a point determined by the V_{max} of the available ASL enzyme. This situation is shown schematically in Fig. 29A. The reverse situation is shown in Fig. 29B. In the latter case the maximal flux per ASL$^-$ cell was only 4-fold greater than in normal cells. The fact that this increase is much less than the 20-fold

FIGURE 29



A.



B.

Quantitative relationships in co-cultures of different proportions of ASS^- (hatched) and ASL^- cells.

increase seen when ASL is the limiting enzyme implies that the V_{max} of ASS is lower than that of ASL in these cells *in vivo*. Thus the rate-limiting step in citrulline metabolism by human fibroblasts is either citrulline transport into the cell, or the ASS reaction. If citrulline transport is rate-limiting, the 4-fold increase in flux per ASL^- cell could reflect transfer of citrulline from ASS^- cells to ASL^- cells. Alternatively, increased removal of argininosuccinate by the excess of ASS^- cells could result in increased flux, since ASS is known to be inhibited by argininosuccinate (Tadaka et al., 1979).

When unlabelled citrulline was added to the medium to give a final citrulline concentration of 0.6 mmol/ℓ, a different metabolic co-operation curve was obtained (Fig. 9). Under these conditions the pathway is operating near its maximal rate (Figs. 3 and 10). The curve of metabolic co-operation as a function of percentage of cell type was less skewed. In terms of the reasoning presented above, this probably reflects the greater saturation of the ASL enzyme under conditions of high pathway flux.

The finding that the maximal rate of citrulline incorporation was as high in the co-cultures as in a culture of normal cells (Fig. 10) indicates that the flux of citrulline to arginine is not limited by the capacity of the intercellular junctions to transmit argininosuccinate. This has implications for the interpretation of the effects of inhibitors of junctional communication (see Section 9.6).

9.4. COMPARISON WITH OTHER SYSTEMS FOR MEASURING METABOLIC CO-OPERATION

Many different model systems using cultured cells have been used to study intercellular communication (see Section 6.1), and these have been recently reviewed (Hooper, 1981). The system presented here has the advantages that the method is simple and rapid to perform, and the results are accurately quantifiable. The use of a second isotope (^{3}H-leucine or ^{3}H-phenylalanine) corrects for errors due to differences in the number of cells in each dish and due to losses of TCA-precipitable material during the washing procedure. Because incorporation of radioactivity occurs only as a result of metabolic co-operation, the background measurement is very low. In addition, the method does not require autoradiography or selective cell-killing techniques which are more difficult methods to quantify. ASS-deficient cells have previously been used in studies of metabolic co-operation (Hooper and Morgan, 1979). These authors used Chinese hamster Don cells which are ASS-deficient and suffer arginine starvation when placed in citrulline-supplemented arginine-free medium. Cells containing ASS are able to relieve the arginine starvation in the Don cells by transferring arginine and/or argininosuccinate via intercellular junctions. It was necessary for arginase and ASL to be present in the medium in order to prevent non-junctional transfer of these metabolites. In the system described here non-junctional transfer is negligible, as shown by the absence of metabolic co-operation when cells which

were not in contact were incubated in a common medium.

The major shortcoming of the method of measuring metabolic co-operation presented in this thesis, is the fact that it depends on the use of two mutant cell lines. It cannot, therefore, be used to study junctional communication in other cultured cells which are not deficient in ASS or ASL. Equally, it has no direct application to the important questions of cell-cell communication in intact tissues, or in the living organism.

Another limitation is the fact that the assay depends on incorporation of citrulline into protein. This means, firstly, that a substantial time must be allowed for isotope incorporation. Using isotopes at high specific activity, the labelling period can be reduced to 1 hour (e.g. Figure 11), but shorter times are not practicable owing to the high cost of the isotopes.

Thus very rapid changes in junctional permeability (occurring over periods less than an hour or so) cannot be resolved without using quantities of ^{14}C-citrulline which are excessively expensive.

Secondly, experimental conditions are somewhat constrained in that they must obviously permit protein synthesis to occur in order to be able to measure citrulline incorporation: it is not possible using this system to test, for example, the effect of protein synthesis inhibitors on junction formation.

9.5. EFFECTS OF EXTRACELLULAR pH, CALCIUM AND MAGNESIUM ON METABOLIC CO-OPERATION

Although not explicitly stated in the literature, variations in extracellular pH within the physiological range have not been reported to affect intercellular junctional communication in other systems. In the system of ASS^-/ASL^- co-cultures used here, metabolic co-operation was not affected by extracellular pH over the range 6.9 to 7.8 over a period of 2 hours (Section 8.10). The implication of this is that the pH of the labelling medium need not be controlled with any more than the usual degree of accuracy, during metabolic co-operation experiments.

Junctional communication has been reported to be modulated by intracellular pH (Spray et al., 1981; Spray et al., 1984; Peracchia et al., 1983; Rose and Rick, 1978; Turin and Warner, 1980). To achieve intracellular acidification these authors used CO_2 or substituted benzyl esters (Spray et al., 1984). It will obviously be of great interest to see what effect intracellular acidification has on ASS^-/ASL^- co-cultures. Such experiments are planned at the time of writing.

The results shown in Table 10 indicate that established permeable junctions between human fibroblasts do not require external divalent cations for their maintenance, at least over a period of 2 hours, provided that the cells are not mechanically disrupted. This confirms quantitatively the finding of Cox et al. (1974) who used a qualitative auto-

radiographic method for estimating metabolic co-operation between human and hamster cells. The resistance of mammalian gap junctions to removal of divalent cations was demonstrated by Berry and Friend (1969) who studied the ultrastructure of liver cells separated by exposure to enzymes and EDTA. Although desmosomes were quickly cleaved, gap junctions persisted intact, retaining small segments of cytoplasm from formerly opposing cells. Similar results were obtained in rat heart by earlier workers (Dreifuss et al., 1966; Muir, 1967). In our cultures, removal of divalent cations markedly inhibited cell-substratum adhesion, as shown by the rounding up of the cells, so that adjacent cells appeared to be in contact only via thin processes (Fig. 15). Evidently the transfer of ^{14}C-argininosuccinate must occur through these processes and the permeable junctions must be located where processes from adjacent cells meet.

In this respect mammalian cells differ from insect salivary gland cells in which intercellular electrical coupling is extremely sensitive to a reduction in the concentration of extracellular divalent cations (Nakas et al., 1966; Loewenstein et al., 1967; Rose and Loewenstein, 1971).

Formation of new permeable junctions must clearly depend on prior intercellular adhesion, a process which is well-known to depend on divalent cations (Okada et al., 1974). Either calcium or magnesium alone is capable of stimulating intercellular adhesion, but the quantitative relation between the degree of adhesion and the cation concentration

is different for the two cations and also differs among different cell lines (Okada et al., 1974). The curves shown in Fig. 16 probably simply reflect the concentration dependence of the adhesion process in the cells used; however, a specific effect of calcium or magnesium on new permeable junction formation per se cannot be excluded.

Gap junction structures are visible in cultured mammalian cells aggregated in suspension (Johnson and Preus, 1973; Johnson et al., 1974), and electrical coupling and metabolite transfer can be demonstrated between hepatoma cells aggregated in suspension (Lloyd et al., 1976; Pederson et al., 1980). The results shown in Fig. 16 confirm that junctions permeable to larger molecules than those involved in electrical coupling (in this case ^{14}C-argininosuccinate) form between anchorage-dependent cells aggregated without adhesion to the substratum.

The results presented here further demonstrate the usefulness of this system using ASS^- and ASL^- cells in providing precise, quantitative information on intercellular communication under a variety of experimental conditions.

9.6. INHIBITION OF METABOLIC CO-OPERATION BY DDT

The measurement of the degree of intercellular junctional communication used in these experiments requires the conversion of ^{14}C-citrulline to ^{14}C-arginine and subsequent incorporation into TCA-insoluble material. Inhibition of incorporation of citrulline can therefore be caused by a block at any of the following stages in this process: (a) citrulline transport into the cell, (b) conversion to argininosuccinate, (c) passage of argininosuccinate through the intercellular junctions, (d) conversion to arginine and (e) incorporation of arginine into protein. In order to demonstrate a block in intercellular communication (step c), it is necessary to show that none of the other steps in the process of citrulline incorporation are affected by the chemical being tested. This is accomplished by showing that ^{14}C-citrulline incorporation in normal cells is unaffected (or is affected to a lesser degree than in co-cultures) by the test chemical, since the only difference between normal cells and co-cultures is that in the latter, functional intercellular junctions are required to complete the citrulline incorporation pathway.

DDT caused an almost complete (90-98%) block of citrulline incorporation in co-cultures at concentrations which had only a minor effect on citrulline incorporation by normal cells, thus satisfying the above conditions for demonstrating inhibition of intercellular communication (Section 8.13). The fact that DDT, a known inhibitor of intercellular

junctional communication (Kurata et al., 1982; Trosko et al., 1982; Williams et al., 1981) blocks citrulline incorporation in the system described here, validates this method of measuring intercellular junctional communication. The effect of DDT on intercellular communication is not merely the result of toxic effects on cellular metabolism. This is shown by the normal growth of the cells in the presence of DDT, and by the fact that 2,4-dinitrophenol at cytotoxic concentrations did not affect intercellular communication.

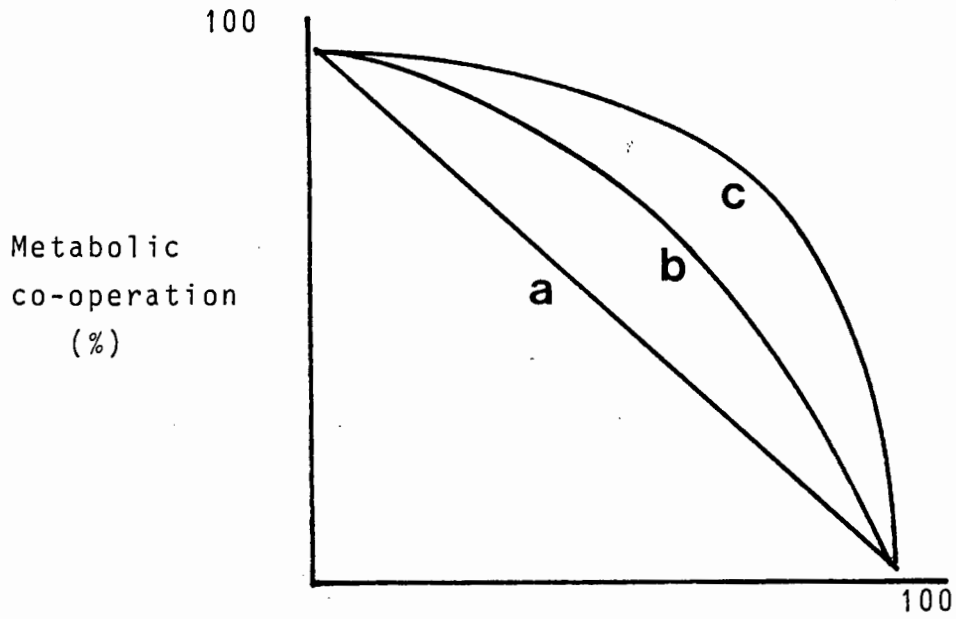
A trivial explanation for the effect of DDT on co-cultures is also theoretically possible: namely that some step in citrulline incorporation is sensitive to DDT in the mutant cells, but not in normal cells. We consider this highly unlikely, since other agents unrelated to DDT also inhibit citrulline incorporation in co-cultures but not in normal cells (Sections 8.15 and 8.16).

The relatively short labelling period of this assay allows the time-course of the effect of metabolic co-operation inhibitors to be determined to a resolution of about 1 hour. DDT was shown to have a rapid onset of action and the block to metabolic co-operation was rapidly reversed by simple washing of the co-cultures. This latter result was surprising in view of the lipophilic nature of DDT, which would be expected to persist in the lipid phase of the cell membrane after washing.

DDT is known to inhibit calcium transport in several systems (Miller et al., 1976; Matsumara and Ghiasuddin, 1979; Ghiasuddin and Matsumara, 1981) and it has been suggested that its inhibitory effect on intercellular communication is due to inhibition of the plasma membrane Ca,Mg-ATPase with consequent rise in intracellular calcium (Madhukar et al., 1983). The fact that the system employed here is not sensitive to removal of extracellular calcium (Section 8.11) enabled this hypothesis to be tested. It was found that the DDT effect was not diminished by removal of extracellular calcium from the medium, indicating that DDT does not inhibit metabolic co-operation by this mechanism. The possibility remains that DDT might increase intracellular free calcium by causing release of calcium from mitochondria or other intracellular stores. Other possible mechanisms are that DDT might alter the properties of the lipid portion of the junctional membrane or that DDT might bind to the hydrophobic parts of the gap junction protein in the membrane. Both of the above mechanisms could lead to a conformational change in the gap junction subunits, thereby blocking the channel. DDT is known to affect a variety of membrane transport systems (Arhem and Frankenhaeuser, 1974; Chefurka et al., 1980; Iturri and Wolff, 1982; Jainicki and Kinter, 1971) and to alter the properties of lipid bilayer membranes (Buff et al., 1982; Wolff and Bull, 1982). It is also possible that DDT may act by binding to a receptor on the cell surface and thereby initiate an as yet undefined sequence of events leading to gap junction closure.

Although this system of measuring junctional communication provides quantitative information, the numerical relation of the parameter being measured (i.e. citrulline incorporation) to the closure of junctions is unknown. This point is illustrated in Fig. 30. The simplest case would be a linear relationship between metabolic co-operation and junctional blockade (line a). As discussed in Section 9.3, the rate of citrulline incorporation is not limited by the junctions, even under conditions of maximal flux. This suggests that there may be some degree of redundancy of junctions in this system, which could give rise to curves of type b or c of Fig. 30. In the extreme case, curve c, the result is a "threshold" relationship, where metabolic co-operation is unaffected until a certain proportion of channels is blocked. Such a threshold effect would tend to compress the concentration range over which a compound inhibits metabolic co-operation. It is possible, therefore, that DDT acts on intercellular junctions at doses lower than those at which measurable inhibition of metabolic co-operation is observed.

FIGURE 30



Theoretical possible relationships between inhibition of metabolic co-operation and degree of junctional blockade.

9.7. INHIBITION OF METABOLIC CO-OPERATION BY TPA AND RETINOIDS

The inhibition by TPA of citrulline incorporation in co-cultures of the mutant cells, but not in normal cells indicates that TPA inhibits junctional communication in these co-cultures. This effect of TPA has been observed by other workers using several different systems for measuring junctional communication (Murray and Fitzgerald, 1979; Yotti et al., 1979; Fitzgerald and Murray, 1980; Newbold and Amos, 1981; Enomoto et al., 1981; Warren et al., 1981; Guy et al., 1981; Friedman and Steinberg, 1982; Mosser and Bols, 1982; Trosko et al., 1982; Fitzgerald et al., 1983; Walder and Lutzelschwab, 1984).

The two types of co-culture used in these experiments differed in their sensitivity to TPA: complete inhibition was observed in V79/ASL$^-$ co-cultures but only partial inhibition in ASS$^-$/ASL$^-$ co-cultures. Variation among cell types in sensitivity to this effect of TPA has been reported previously, using other methods for measuring junctional communication. Warren et al. (1981) used the "kiss of death" method (see Section 6.1) to demonstrate inhibition of metabolic co-operation by TPA. They found that TPA inhibited metabolic co-operation in V79 cells, but not in CHO cells, and ascribed this difference to cell membrane differences between the two Chinese hamster cell types. Mosser and Bols (1982) found, using an autoradiographic method, that junctional communication in human

fibroblasts was only partially inhibited by TPA, in contrast to the complete inhibition observed with other cell types.

The concentration range over which TPA inhibits junctional communication (0.1 to 10 ng/ml) is similar in this system to that found using other methods such as direct microinjection of dye into cells (Fitzgerald et al., 1983) or the survival of hypoxanthine-guanine phosphoribosyl transferase-deficient cells in 6-thioguanine (Yotti et al., 1979; Trosko et al., 1982). Over the same concentration range TPA produces many other effects on cells (reviewed in Diamond et al., 1980) and it is thought to act by binding to specific cell surface receptors (Shoyab and Todaro, 1980; Horowitz et al., 1981; Salomon, 1981). It therefore seems likely that inhibition of junctional communication is another consequence of receptor occupancy. Recent evidence suggests that the phorbol-ester receptor is a quaternary complex formed by the phorbol ester, calcium, membrane phospholipids and the Ca^{2+}-sensitive, phospholipid-dependent protein kinase (protein kinase C) (Castagna et al., 1982; Rozengurt et al., 1983; Naka et al., 1983; Nishizuka, 1984). Many, if not all of the responses to phorbol esters may be due to the activation of protein kinase C, with resulting phosphorylation of cellular proteins (Nishizuka, 1984). It is possible that the inhibitory effect of TPA on junctional communication results from the phosphorylation of gap junction subunits, leading to conformational change and closure of the cell-to-cell channel, although it must be emphasized that

there is as yet no direct evidence for or against such a sequence of events.

Metabolic co-operation in both co-culture systems used here became refractory to some degree to the inhibitory effect of TPA after long (19 hour) exposure to high concentrations (100 ng/ml or greater). However, refractoriness did not develop at lower TPA concentrations (1 to 10 ng/ml) (Figure 23). A similar reversal with time of communication inhibition by TPA has been reported in mouse cells (Fitzgerald and Murray, 1980; Guy et al., 1981), in human fibroblasts (Mosser and Bols, 1982) and in rat liver cells (Walder and Lutzelschwab, 1984) using other methods of measurement. Several of the other cellular responses to phorbol esters are also transient, and are followed by a refractory state during which responses to phorbol esters are attenuated (Salomon, 1981; Castagna et al., 1982; Lee and Weinstein, 1979; Magun et al., 1980; Phillips and Jaken, 1983). Down-regulation of phorbol ester receptors (as measured by phorbol ester binding to cells) and of protein kinase C activity in cell extracts has been demonstrated following prolonged exposure to active phorbol esters (Salomon, 1981; Magun et al., 1980; Phillips and Jaken, 1983; Solanki and Slaga, 1982; Rodriguez-Pena and Rozengut, 1984). It therefore seems likely that the refractoriness of junctional communication to TPA after prolonged exposure to high concentrations is due to down-regulation of phorbol ester receptors. In the system of V79/ASL$^-$ co-cultures used here there is a concentration range (1 to 10 ng/ml) at which inhibition of

junctional communication by TPA is maximal but refractoriness does not occur. This suggests that the rate of down-regulation increases progressively with increasing TPA concentration above the level which causes maximal inhibition of junctional communication.

Reversal of inhibition by TPA after washing the cells was slow in comparison with reversal of inhibition by RA (Figure 24) or DDT (Figure 20). This does not necessarily imply that the cellular effect persists after removal of TPA: this compound is highly hydrophobic and the slow reversal observed could be due to persistence of TPA in cell membranes despite washing. A more rapid reversal of the TPA effect has been reported using electrical coupling to measure communication inhibition (Kanno et al., 1984).

Retinoic acid inhibited junctional communication at concentrations in the range 10^{-7} to 10^{-5} M, a thousand fold higher than for TPA. Retinol and retinyl acetate were less potent inhibitors. The inhibition of junctional communication by RA also differed from TPA in three other respects: refractoriness after prolonged exposure to high concentrations of RA did not occur, and the two types of co-culture, which showed a marked difference in sensitivity to TPA, were equally sensitive to RA. Finally, co-cultures which had been rendered refractory to TPA by prolonged exposure to high concentrations, were still responsive to inhibition by RA. These observations strongly suggest that TPA and

retinoids inhibit junctional communication by different mechanisms. The concentrations of retinoids required to cause 50% inhibition of junctional communication were at most ten fold less than cytotoxic concentrations (Table 16). This suggests that inhibition of junctional communication by retinoids may be due to direct alteration of membrane physical properties by intercalation of the retinoids into the membrane. RA is reported to stimulate gap junction formation in chicken epithelium (Elias and Friend, 1976) and in skin tumors (Prutkin, 1975; Elias et al., 1980). The relationship of this phenomenon to RA-induced inhibition of junctional communication is not clear, but it has been suggested that the gap junction proliferation may be a response by some feedback mechanism to decreased junctional communication (Pitts et al., 1982).

Co-cultures which had been made refractory to TPA retained their sensitivity to inhibition by DDT: this is strong evidence that DDT, like RA, inhibits junctional communication by a mechanism different from phorbol esters.

The quantitative nature of the assay system used here to measure junctional communication facilitated a study of the interaction between TPA and RA. The experiments in which co-cultures were exposed to TPA and RA simultaneously failed to show any antagonism between the two compounds in regard to their effects on junctional communication. The inhibitory effects of the two compounds were additive. The

potent anti-tumor-promoting agent FA also failed to antagonise the inhibitory effect of TPA on junctional communication. These results suggest that retinoids and FA exert their anti-tumor-promoting effects by mechanisms which are not mediated by junctional communication.

9.8. CONCLUSIONS

The quantitative nature of the information provided by this assay system should facilitate a comparison between different compounds which inhibit intercellular communication. In this way it may be possible to define the molecular structure responsible for the inhibition, and to determine whether there are compounds which specifically inhibit intercellular communication, without affecting other cellular functions. In view of the relationship which is becoming apparent between tumor-promoting activity and inhibition of intercellular communication, the assay described here should prove useful in the screening of suspected tumor-promoting agents.

It is possible, using this system, to manipulate the experimental conditions in a variety of ways: the composition of the media can be changed, and the effects of biologically active compounds can be tested. In this way the system could be used to elucidate the mechanisms of action of inhibitors of junctional communication, and to gain insight into factors which may regulate junctional permeability physiologically. This knowledge may in turn throw light on the biological roles of these ubiquitous cell-to-cell channels, as well as on the mechanisms of action of tumor promoters.

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